

# The technology of Wednesday's war

If there is a war in the Gulf next week, there will be no winners, only losers. Would it not be prudent to begin now on planning what might become of the Middle East when all the dust has settled?

WHETHER the impending conflict over Kuwait begins next Wednesday (as advertised), on some later date or (preferably) never, the Middle East will never again be the same. Most probably the stability and civility of this tinder-box region will not be improved, but rather, worsened by a shooting war — as they have been by the preliminaries. All kinds of things can go wrong in the weeks ahead. As with all wars, the pointless loss of life is the chief calamity, which dwarfs all others. But there are other hazards to watch out for in the weeks ahead, many of them technical in complexion.

A latecomer to the roster of calamity is the notion that Iraq, faced with the military defeat that seems on the cards, might set Kuwait's oilfields on fire and thus ordain the burning of 10 million barrels of oil a day for the next year or so (see page 96). Not since the late Sir Robert Robinson thought it worth warning the British Association for the Advancement of Science that hydrogen bomb tests might ignite the oceans, has there been such a vivid picture of how the planet might be set alight. But it is unreal. Putting a torch to the oil wells in Kuwait would certainly add to the global warming supposed to be the likely consequence of the addition of carbon dioxide to the atmosphere. Others argue that the burning oilfields would also put soot into the atmosphere, reducing the input of solar radiation to the Earth's surface in a kind of simulation of nuclear winter.

Both calamitous scenarios depend for their capacity to compel the imaginations of others on a misconception of what an underground oil reservoir is like. Well-heads set alight will not burn indefinitely, but instead will destroy themselves. And even the richest reservoirs are not subterranean lakes of crude oil, but rather are rock strata laden with the stuff. Such reservoirs could be set on fire, but the rate at which they burned would be determined by the rate at which they were aereated. A smouldering near-surface oilfield would be a great nuisance, and would no doubt cause long-term environmental damage if, after a war, no way could be found of containing it. But the calamities that matter are more immediate, although their consequences are likely also to cast long shadows.

What, for example, if some participant in the impending war decides that its strategic interests require the use of nuclear weapons? That is not far-fetched. There are good reasons to believe that Iraq, whatever its ambitions, is not yet a nuclear power, but the United States

has said that it will retaliate massively against any Iraqi use of "weapons of mass destruction" (supposed to be a reference to chemical weapons). Does that mean the US would use its own chemical weapons or nuclear weapons (which would be much more convenient in the circumstances)? Israel, not now a participant, could become one overnight if the president of Iraq means what he has said on several occasions, that his real discontent is not with Kuwait but Palestine (which can be variously defined); Israel might well use nuclear bombs against population centres in Iraq if it were itself gratuitously attacked. But any of the participants in the planned war would probably be able to justify its use of tactical nuclear weapons if, after a few weeks, the military going against Iraqi forces proved unexpectedly rough. They would probably discover that nuclear weapons are just as effective against tank and troop concentrations as generals in the European theatre have been saying since 1960.

## Bad example

That is a demonstration that the rest of us can do without; its effects on other quasi-nuclear powers would be disastrous. Why should countries such as Pakistan hold back on the development of nuclear weapons if the world's registered nuclear powers have already laid on a real-life demonstration of their utility in the battlefields of the Middle East? That is one reason for hoping that the weeks ahead do not occasion the perception by any of the participants that the time has come to use nuclear weapons. Another, of course, is that any such development would further sour relations between the states of the Middle East and between them and the outside world, making a civilized settlement even more difficult to attain than it seems to be at present.

Sadly, even a conventional war is bound to be a telling demonstration of the firepower of modern munitions and their delivery systems — missiles, aircraft and old-fashioned gun barrels. The last major war involving the United States, in Vietnam, was fought with weapons designed a quarter of a century ago. The funds since then spent on military research and development by the industrialized powers might preferably have been spent on other things, but they have not been wasted. If there is a war next Wednesday, it is likely to be awesome proof of that. Afterwards, many smaller countries will be left with the conviction that they must re-equip themselves, no



doubt at ruinous cost. That will also prove to be a demonstration that we could have done without.

If there is a war, it will be (like that in Vietnam) a television war. The government of Iraq during the past four months has skilfully provided facilities for visting camera crews, evidently recognizing the importance of showing itself to be a country full of ordinary people. No doubt the facilities will continue to be provided if war breaks out, with the consequence that the same ordinary people will frequently be shown to have been killed. And the television news reports will similarly record injuries and death among the forces ranged against Iraq. The Vietnam experience in the United States showed that the capacity of democratic populations to absorb a diet of such graphic horror is strictly limited. The technology of television communications could be the President of Iraq's best hope of bringing a losing war quickly to a halt, but with the further benefit of spreading dissension and provoking recrimination among those who are arrayed against him.

That would be a great shame, not least because it would challenge the new-found authority of the United Nations, marvellously strengthened by events in the past two years and full of promise for the general improvement in years ahead. That, rather than the national pride of any participant, is the chief reason why an unwelcome war may have to be fought. But that should not imply that there is no room for regret. There seems, for example, general agreement that the Middle East will need some more durable way of conducting its affairs in the decades ahead. It does not make sense that one of the richest regions of the world should also be one of the poorest, both in natural resources other than petroleum, and in the poverty of its armies of migrant workers.

It is natural that the political problems of the region should have been put on ice during the past five months, especially because Iraq has linked the settlement of some of them with its compliance with the resolutions of the UN Security Council, but that is no reason why schemes for making the Middle East a better place in which to live should also have been frozen. And even now, it is not too late. No harm would be done if people began now to plan ways of beating into ploughshares of an appropriate kind technology such as that arrayed against Iraq in the Saudi desert. The successful prosecution of a war may require single-mindedness, but those who suffer most from war also rightly expect their governments to pay attention to the more distant future. □

## What to tell Major

Britain's new prime minister will be told next week what needs to be done for British science.

SIR Ian Lloyd, the British Conservative Member of Parliament with a distinguished record as a back-bencher and as chairman of House of Commons committees, has hit on

an old-fashioned but neat device for drawing the new Prime Minister's attention to the plight of the British research enterprise: he plans to go to see him next week, in the company of people such as the recently retired president of the Royal Society and the vice-chancellor of the University of London. The device is neat because it is potentially disarming. But it is also potentially frustrating; time can be frittered away by pleasantries so that even deputations as distinguished as next week's may find themselves at the door on the way out before they have said their pieces, and be compelled to leave their arguments in written form for civil servants to mull over. But two things need saying loud and clear.

### Needs

First, British science needs ministerial representation. It is an old argument (whose force was acknowledged when, in the 1960s, it was practised for a time). Ministerial representation does not mean that more money must be spent, but merely that connected thought should be given to public support for the research enterprise, and public use thereof. The argument was last made by the House of Lords Select Committee on Science and Technology in 1981, but then avoided by the previous prime minister, who accepted that something should be done but said that, being a scientist, she would do the job herself. A debating point for next week is the observation that, now she has gone, the once-accepted need becomes more clamant.

Second, there is an urgent need that the new British government should disown the naive view of the relationship between research and industrial innovation which its predecessor embraced and helped put into circulation. This is the opinion that, in economically unsuccessful countries such as Britain, research and development (otherwise R&D) are separable from each other, that D is virtuous (and deserves government support), but that R is a luxury whose benefits (if any) will be long-delayed. Just this view was put out at the end of last year by one of Britain's otherwise better newspapers, the *Financial Times* (14 December 1990).

This misconception, sustained by an over-simple reading of the recent industrial history of Japan, has been generally promulgated by British governments since 1964, and is now widely shared by British industry, still woefully unaware of the benefits it derives from its recruitment of young people trained in research at British universities and polytechnics. Until some government acknowledges that R&D are related symbiotically, and that the most immediate contribution of R to D is skill (bright ideas take longer), this antediluvian opinion will persist. Sadly, the manpower problem in Britain is now so serious that it will take years of connected thought to put it right (which is again where ministerial representation could help). Meanwhile, next week's deputation might persuade Mr John Major, who seems willing to please, to cut the occasional tape at the occasional opening ceremony. □

# US challenge to AZT patent

- AIDS treatment too costly
- AZT discovered in US lab

## Washington

THE patent for AZT, at present the only drug licensed for the treatment of AIDS, is being challenged by the US government and two Canadian companies in an attempt to wrest it from the hands of Burroughs Wellcome, the British pharmaceutical company that has made a fortune from it.

At issue is the price of the drug, and the profits Burroughs has taken from its sale. Three years ago, when Burroughs first introduced AZT, it charged more than \$3.00 per capsule. For the average AIDS patient, that meant an annual cost of some \$10,000. Under pressure from AIDS activists, the company has since cut the price by two-thirds, to \$1.20 per capsule, but critics say it is still overpriced.

Because medical costs quickly impoverish many people with AIDS, government subsidies usually end up paying for most of the AZT. So far, those subsidies have amounted to some \$420 million of the \$700 million spent on the drug. Much of that money, critics say, could have gone to research instead.

Burroughs has refused publicly to disclose the cost of making AZT, saying only that research and development has cost the company "hundreds of millions of dollars" and that the price of the drug reflects that. But US health officials and congressional investigators are now disputing what Burroughs says: they say that government scientists at the National Cancer Institute (NCI) first discovered AZT more than two decades ago, and then in 1985 proved it killed the human AIDS virus.

Burroughs' major contribution, according to NCI researchers, was to pick AZT — at that time a failed cancer drug — among some four dozen other compounds in the public domain to be tested in collaboration with NCI for possible AIDS applications. AZT turned out to be surprisingly effective.

NCI also turned over its supply of an essential and rare compound — thymidine, which is found naturally only in herring sperm — so that Burroughs could produce the AZT. In 1985, Burroughs won an exclusive patent on the drug. There was no mention of the role of the US scientists.

Among other options, lawyers at the US National Institutes of Health are considering challenging the patent on the grounds that the US government should have been listed as a co-inventor, accord-

ing to a spokesman. The American Civil Liberties Union and Public Citizen, a non-profit litigation group, are also considering legal action. And, as first reported by the US television network ABC last week, two Canadian companies — Apotex Inc. and Novopharm — have challenged the Burroughs patent in Canada.

Apotex already has large quantities of AZT and has begun shipping it for \$0.89 per capsule — two-thirds of the Burroughs price — to countries such as the Bahamas, where Burroughs does not have a patent. Canadian researchers believe they can make AZT as cheaply as \$0.50 per capsule.

"We are virtually certain we will win", says Apotex president Barry Sherman. "There was no invention, and even if it were an invention, [Burroughs] was not the inventor. If a product in the public domain proves useful for AIDS, that is a scientific effect, not an invention." He expects similar patent challenges in the United States within months. A Burroughs spokeswoman said only that "we are confident in our patent."

The large fraction of government AIDS money that has gone to AZT has drawn the attention of Congressman Ted Weiss (Democrat, New York), head of the House of Representatives human resources investigative subcommittee. Although initially spurned in his request for an accounting of Burroughs' AZT costs, Weiss scored a victory last month when the company provided the figures under a promise of confidentiality. Weiss's staff is now analysing those figures and may hold a hearing, but Weiss has already made his mind up on one point: AZT costs too much and the fault lies both with the government and with Burroughs.

"For several years the federal government did nothing to enforce its ownership right to AZT", Weiss says. "Government inaction allowed Burroughs Wellcome to reap excessive profits from persons suffering from AIDS who need the drug to lengthen their lives. There will be no reason for Burroughs Wellcome to lower the inordinately high price of AZT until the federal government exerts its rights, including a share in past profits".

Although the onslaught seems sure to wrest concessions — if not outright control of the patent — from Burroughs, the news may not be all good for the AIDS community. The price of AZT is likely to drop, from competition if nothing else,

but the strong-arm tactics used by activists and legislators alike may send a worrying signal to the rest of the industry. "You can't go too far; you don't want to discourage companies from getting involved in AIDS-related research", warns Pat Christen, director of the San Francisco AIDS Foundation. Burroughs (or the company that ends up manufacturing the drug once the patent disputes are resolved) must be allowed a fair profit. Just how much profit is fair, and who should decide, remain the pressing questions.

**Christopher Anderson**

## ASTRONOMER ROYAL

### New man will speak his mind

#### London

ARNOLD Wolfendale, professor of physics at the University of Durham, is Britain's new Astronomer Royal — an honorary position dating back to the seventeenth century. A vocal critic of the low British spending on astronomy and space science, Wolfendale promises that his official appointment will not prevent him from speaking out in future: "I may need to be more circumspect, but the message will be the same".

Wolfendale's research centres on cosmic rays and high-energy solar particles. He replaces the radioastronomer Sir Francis Graham-Smith, who resigned the post after his retirement from the University of Manchester.

Wolfendale has been chairman of the Astronomy and Planetary Science Board of the Science and Engineering Research Council (SERC) since 1988. British astronomers hope that the outspoken Wolfendale will fight their corner as support for 'big science' projects comes under scrutiny in the council's search for ways to release more money for small project grants.

Last month, the usually apolitical Royal Astronomical Society voiced its concern about the damage to the "first-class" British presence in astronomy and space physics that would result from any reduction in support. Astronomy has borne the brunt of SERC's immediate cost-cutting to prevent a cash shortfall of £40 million in 1991-92: two important projects — a collaboration with the United States and Canada to build two 8-metre optical telescopes, and the British contribution to a gravity wave observatory to be built in Germany — have been delayed for at least two years.

The post of Astronomer Royal has had no set duties since the link with the directorship of the Royal Greenwich Observatory was severed in the early 1970s, but the Astronomer Royal is meant to represent the British astronomical community.

**Peter Aldhouse**



# Huge increase for global environment?

## Tokyo

INCREASED spending on research projects linked with the global environment stands out from the Japanese government's research budget for the next financial year, which begins on 1 April. Compared with the usually small yearly changes, some of the planned increases are dramatic.

Both the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency (STA) have won substantial new funds for research on space and the global environment. The budget, now approved by the cabinet, has yet to be approved by the Diet, but the allocations to science and technology are unlikely to be changed.

Although MITI's overall research and development budget has been held to a modest increase of only 2.6 per cent — barely enough to keep pace with inflation — it will be able to increase the funds available for research by using special accounts derived, for example, from taxes on oil and electricity. On the other hand, STA, which has a research budget twice as large, gets a healthy boost of 5.6 per cent.

MITI's problems arise because its general account has to decrease by 10 per cent each year under strict Ministry of Finance rules aimed at curbing government expenditures. STA's general account is not subject to such severe limits.

Most of MITI's spending of about \$50 million on the global environment — up nearly 20 per cent on last year — will go to

government/industry projects to develop environmentally friendly technology such as substitute CFCs and the fixation and utilization of carbon dioxide. These projects will be coordinated by MITI's new Research Institute of Innovative Technology for the Earth (RITE) when that opens, in 1992, in the Kansai science city between Osaka, Nara and Kyoto. As well as MITI's direct support, RITE has won funding of ¥8,000 million from local governments and industry, making it probably the largest project ever launched by MITI.

There is also an enormous increase for STA's 'Green Planet' project, aimed at observing and monitoring the Earth's environment. More than 50 per cent of the project's budget, or ¥5,737 million, will go to the development of the Advanced Earth Observation Satellite (ADEOS), due to be launched in 1995. There is also a significant increase in the agency's funds for ocean research, including a joint project with the US Woods Hole Oceanographic Institution to study the Northern Pacific and Arctic Oceans.

Apart from the new satellite, the main reason for the increase in STA's budget for space is a dramatic increase in outlays for Japanese participation in the US space station. The agency's space station budget almost doubles to ¥17,950 million (\$135 million) this year.

MITI's budget is also brightened by a 10 per cent increase in funds for the Japan

Key Technology Centre, operated jointly with the Ministry of Posts and Telecommunications (MPT). The centre is funded with the dividends of government-held shares of the recently privatized Nippon Telegraph and Telephone Corporation (NTT). Two of the key projects the centre supports are MITI's Protein Engineering Research Institute in Osaka and MPT's Advanced Telecommunications Research Institute (ATR) in Kansai science city, which are among the best financed and best equipped research institutes in Japan.

Ironically, the fall in the value of NTT shares last year in the stock market slump has brought about this year's increase of funds for the centre. The government put pressure on NTT to increase its dividend payments to compensate shareholders for the enormous loss in value of their shares. But

MITI officials fear that the increased dividends will be just a one-off event.

Both MITI and STA continue to pour extra money into international research projects. A MITI project to develop a hypersonic commercial aircraft in collaboration with European and US aerospace companies, including Rolls-Royce of the United Kingdom and Pratt and Whitney of the United States, gets more than 20 per cent extra, or ¥6,700 million (\$50 million) next year. A final agreement covering intellectual property rights for the project is expected shortly.

Both MITI and STA have increased their budgets for the Human Frontier Science Program, which distributes grants and fellowships to international teams for research on the brain and molecular biology. MITI's Intelligent Manufacturing System project to develop the automated factories of the future gets extra funds for an international feasibility study.

The budget for STA's ERATO projects continues to grow rapidly. And the agency this year will launch a new project, Sakigake Research 21, modelled on ERATO, that will disperse grants to individual researchers rather than teams.

Twelve individuals will be selected in 1991 and will each receive an average of ¥60 million (\$450,000) over three years to support research on bioscience, material science and electronics. Similarly, MITI is expanding its budget for a programme modelled on the Human Frontier project that distributes domestic grants for elucidation of biological functions.

David Swinbanks

## ACADEMIC FREEDOM

### Back to flesh

#### Washington

It may not seem much to crow about, but Canadian researcher Philippe Rushton reports that he will now be allowed to teach his classes in person. For the embattled psychologist, whose controversial work on race, sexuality and intelligence has drawn wide protest in Canada, permission to face his students means the end of a semester of videotape presentations.

Pre-taped lectures have been Rushton's only classroom appearances since university officials, fearful of demonstrations, banished him from live teaching last year (see *Nature* 347, 6; 1990). Perhaps for just that reason, demonstrations did not become a major problem, and in the name of academic freedom, some students and faculty rallied behind him.

The end, however, may not yet be in sight. Rushton also reports that he is hard at work on a book covering the subjects that earned him a vice squad investigation last year. "It would be naive to believe that it will be smooth sailing from now on", he observes.

Christopher Anderson

#### JAPANESE SCIENCE BUDGET FOR 1991

##### Ministry of International Trade and Industry (MITI)

|                                          | Thousand million yen | % Change from 1990 |
|------------------------------------------|----------------------|--------------------|
| Total R & D budget                       | 258.1                | +2.6               |
| Japan Key Technology Centre              | 28.6*                | +10.0              |
| Basic technologies for future industries | 7.9                  | +5.4               |
| Large-scale industrial projects          | 14.3                 | +1.8               |
| International aircraft development       | 6.7                  | +21.8              |
| Sunshine project                         | 29.9                 | -24.5              |
| Moonlight project                        | 11.9                 | +2.4               |
| Unmanned space platform                  | 9.9                  | +20.7              |
| Fifth-generation project                 | 7.2                  | +2.9               |
| Sixth-generation computer                | 0.1                  | —                  |
| Industrial standardization               | 1.0                  | +9.0               |
| Global environment                       | 7.2                  | +17.4              |
| IMS                                      | 0.3                  | +145.4             |
| Elucidation of biological functions      | 0.3                  | +18.3              |

##### Science and Technology Agency (STA)

|                                |             |        |
|--------------------------------|-------------|--------|
| Total R & D budget             | 522.6       | +5.6   |
| Special promotion funds        | 10.5        | +2.9   |
| Space                          | 131.8       | +10.4  |
| Nuclear energy (ITER)          | 306.8 (2.4) | +3.4 — |
| SOR (Spring-8)                 | 4.9         | +75.0  |
| Ocean research                 | 10.7        | +8.1   |
| ERATO                          | 5.7         | +11.8  |
| Human Frontier Science Program | 3.7†        | +15.7  |
| Green Planet Project           | 10.3        | +119.1 |
| Sakigake Research 21           | 0.5         | —      |

\* Budget shared with Ministry of Posts and Telecommunications

† includes 1,511 million yen from MITI budget

# Precedent set as NIH loses court battle

## Washington

IN a decision that threatens a host of prominent scientific misconduct investigations, a federal judge has ruled that the Public Health Service's existing mechanisms for policing research are illegally constituted and so are invalid.

Although, by early this week, federal officials had not yet announced their response, the ruling appears to set a precedent that could halt all 60-odd misconduct investigations now under way at the National Institutes of Health (NIH).

The decision, by Wisconsin district judge Barbara Crabb, is on a portion of the closely watched case of University of Wisconsin researcher James Abbs, who has been under investigation since 1987 by the NIH for allegedly fabricating data. (Certain curves in an Abbs *Neurology* paper are alleged to have been traced from one of his previous papers on different patients.)

Abbs claims that the protracted investigation has violated his 'due-process' rights, which include the right to cross-examine witnesses, to examine evidence and to be informed of all charges. Abbs also claims that the misconduct procedures of the Public Health Service (of which NIH are a part) had been illegally implemented without the public comment and review period required of federal regulations.

Although the judge decided that Abbs did not have the legal basis to sue for due-process protection (among other things, he could not convincingly show that the investigation had prevented him from continuing his work), she was certainly no more sympathetic towards the government.

"The Public Health Service is not exempt from the requirements of the Administrative Procedures Act. It is required to promulgate agency rules according to the Act's provisions, which include publishing notice of any proposed rules in order to invite comment from those persons who are likely to be regulated by the rules the agency seeks to adopt", the judge wrote in her 28 December decision.

Until the Public Health Service releases its regulations for public comment and review (a process that can take from months to years), they are "invalid", Judge Crabb ruled. Although the decision only makes the NIH illegal in Wisconsin's Western District, it sets a strong legal precedent that can be used as the basis of a dismissal suit by any other non-government researcher currently subject to an NIH probe. (Government employees under investigation, like embattled AIDS researcher Robert Gallo, are not entitled

to the same protection.)

"Unless NIH acquiesces nationally, you will see a torrent of lawsuits," says attorney Robert Charrow of the Washington law firm Crowell and Moring. Assuming that NIH concedes that its rules are illegal and will probably be found invalid whenever they are challenged, the agency will probably have to choose between two unpleasant options, Charrow says. NIH can either offer researchers currently under investigation a one-to-two-year wait while the agency proposes new regulations and has them approved, or offer to convert their cases immediately into formal department proceeding, where full due process rights are already in place. Although the latter ensures prompt court-like treatment, researchers

CONFLICT OF INTEREST

## Then there were four . . .

### Washington

US health officials have revealed that yet another colleague of AIDS researcher Robert Gallo is under investigation, bringing the total to four inquiries in the past year at Gallo's laboratory.

In a confidential letter released last week by congressional investigators, the National Cancer Institute (NCI) reported that Prem Sarin, now second-in-command at the laboratory, has been removed from his position and is facing suspension for possible conflict-of-interest violations.

NCI officials are focusing on the possibility that in 1987 Sarin took money from two drug companies, one of which made an anti-AIDS drug that was being tested at the laboratory. "Based on the very serious nature of the possible violations, on 21 December, [Sarin] was removed from his position as Deputy Chief, Laboratory of Tumor Cell Biology, and reassigned to a non-supervisory, non-managerial position", the NCI letter states.

Sarin has also been given two weeks to respond to a proposal to suspend him from all duties without pay until the outcome of an NCI investigation. His lawyer has denied the allegations of wrongdoing and promised full cooperation.

The NCI decision comes in the wake of a six-month investigation by the staff of congressional watchdog Representative John Dingell (Democrat, Michigan). Sarin had been one of the witnesses at a hearing Dingell called last year to review the actions of another Gallo associate, Syed Salahuddin, who was later convicted of steering NCI funds to a business of which he was a part-owner.

found guilty in such a proceeding can be barred for life from obtaining government grants without an opportunity for further appeal. Such a proceeding normally only comes after a full NIH investigation has found evidence of misconduct.

Officials at the NIH Office of Scientific Integrity (OSI), which handles misconduct investigations for the agency, were not available for comment.

OSI has been under increasing fire for its lack of due process protections (see *Nature* 346 9; 1990), and NIH officials have privately worried that the entire misconduct process is vulnerable to legal challenge, a fear that now appears to have been borne out with the first courtroom test of the system. Should NIH be forced to halt all current activities and start again, scientists now under investigation can expect at least another year in limbo while NIH scramble to rebuild their misconduct machine.

Christopher Anderson

Although Sarin had not expected to be a target of that inquiry, Dingell launched a surprise attack at the hearing by questioning him at length about his representation of a Wisconsin company at a 1986 Food and Drug Administration meeting (see *Nature* 345, 99; 1990).

Since then, Dingell's staff have subpoenaed Sarin's personal bank records, NCI files and financial information from the two drug companies in an attempt to document their suspicions of illegal conflicts of interest. It was in response to one such request that NCI officials reviewed the records themselves and decided to remove Sarin from his post.

Beyond the laboratory connection, there is apparently no link between the Sarin and Salahuddin investigations, or with the continuing investigations of Gallo and his former chief virologist Mikulas Popovic for possible misconduct related to the discovery of the AIDS vaccine.

Salahuddin was last year found guilty and sentenced to repay \$12,000 and carry out 1,750 hours of research as a community service. While the Gallo and Popovic investigations are still months away from any conclusion, Dingell continues to show his interest in the case by investigating others of Gallo's colleagues.

Whether the high number of questionable dealings that the Dingell staff has uncovered reflects a chance cluster of wrongdoing, an unusually low ethical standard in Gallo's laboratory or a fundamental clash between common scientific practice and federal regulations will no doubt be on the agenda of one of several hearings expected this year.

Christopher Anderson



# Oil-well climate catastrophe?

## London

THE British Meteorological Office is assessing the climatological impact of a war in the Persian Gulf, after delegates to a hastily convened conference in London last week suggested that Iraqi sabotage of Kuwait's oil wells could cause an environmental catastrophe.

The fears expressed last week centred around the cloud of soot that would result if Kuwait's oil wells were set alight by Iraqi forces, and the possibility that this could block out sunlight, with effects similar to those of the 'nuclear winter' much discussed some years ago as a consequence of an all-out nuclear war.

John Houghton, director of the Meteorological Office, hopes to have a preliminary climatological analysis later this week, before the 15 January deadline set by the UN Security Council for Iraqi forces to leave Kuwait. "We don't think there's a major climate problem", he says, but adds that "it is something we have to look at", given the publicity attracted by the London conference.

Before the Iraqi invasion, Kuwait produced more than two million barrels of oil a day from 365 active wells, of which 343 required no artificial pumping and so could flow freely (probably at a slightly higher rate than when controlled). The fear is that the wells would continue to burn if they were ignited. Given that there are only a handful of specialist oil-well fire-fighting teams around the world, it could take from six months to a year to extinguish all the fires if most Kuwaiti wells were sabotaged.

At the London meeting, John Cox, a consultant chemical engineer to the oil industry who is also a vice-president of the British organization Campaign for Nuclear Disarmament, said 3 million barrels of oil a day could burn if all the Kuwaiti wells were sabotaged. Abdullah Toukan, science adviser to King Hussein of Jordan, also attended the London meeting and says that as much as 10 million barrels of oil a day could burn if a proportion of Kuwait's many out-of-commission oil wells were also set alight. But Iraq may be deterred from such widespread sabotage by the fact that the worst environmental effects would be felt in the Gulf region, including Iraq.

The Meteorological Office study has been requested by a number of government departments. Keith Browning, director of research, says that there is too little time to run any detailed computer simulations, and is aiming at a short "common-sense assessment". Browning's calculations are based on the burning of 2 million barrels of oil a day if most wells are sabotaged, but his team will also produce analyses based on partial sabotage of the

Kuwaiti oil fields and for a worst-case situation.

Taking Toukan's figure of 10 million barrels a day, Paul Crutzen, from the Max Planck Institute for Chemistry in Mainz, has produced some rough calculations which predict a cloud of soot covering half of the Northern Hemisphere within 100 days. Crutzen stresses that he cannot vouch for the accuracy of the Jordanian figure, but estimates that temperatures beneath such a cloud could be reduced by 5–10 °C in the short term.

But this tentative forecast requires that soot particles should reach the stratosphere if they are to have anything other than a regional effect. Some climate scientists doubt whether the blazing oil wells could create sufficiently strong convection currents to deposit soot into the upper atmosphere.

The problem for the Meteorological Office team is that very little work has been done on the circulation patterns set up by large fires. Martin Miller, from the European Centre for Medium-Range Weather Forecasting in Berkshire, who works on convection currents in natural weather systems, says that there has been some work on forest fires, but "it's a very imprecise science". David Shillito, from the consultant chemical engineers Cremer and Warner, adds that a detailed climatological assessment of the sabotage would require data on the likely yield of carbon from the fires, and on the size distribution of soot particles — parameters that may vary from well to well.

Last week's conference was organized by Penny Kemp, a writer on environmental issues, and was attended by a range of interested parties, including environmental engineers, representatives of the oil industry and peace campaigners. Kemp says that a similar meeting will be held in the United States this week. The warnings of global disaster have been featured prominently by the British media, but were dismissed as "misleading" by the Secretary of State for Energy in the British government, John Wakeham.

The possibility of an environmental catastrophe in the Gulf was first raised by King Hussein in his speech to the World Climate Conference in Geneva last November, when he said global warming could be accelerated by the burning of Kuwait's oil reserves. But many climate scientists say that the sabotage of Kuwait's oil wells, against the background of worldwide fossil fuel burning, would have only a small warming effect. Tom Wigley, from the University of East Anglia, says he would expect sabotage of Kuwait's oil wells to produce only a small "blip" in the global atmospheric carbon dioxide concentration.

**Peter Aldhous**

# First winner in detector race

## San Francisco

THE Superconducting Super Collider (SSC) Laboratory last week announced which two of three contending groups may build \$500-million particle detectors for the accelerator. The results: one clear winner, one group out of the running and one asked to try again after revamping its collaboration.

The Solenoidal Detector Collaboration (SDC), led by George Trilling of the University of California's Lawrence Berkeley Laboratory, was given the go-ahead to develop a formal design proposal, to no great surprise in the high-energy physics community. The SDC combines a proven technological approach with respected leadership, and was the only proposal with the broad general-purpose capability for tracking particles that the project requires of one of the two large detectors.

The youngest and smallest collaboration to propose a detector was EMPACT/TEXAS, headed by Michael Marx of the State University of New York at Stony Brook. This scheme has been turned down by the SSC's Program Advisory Committee (PAC), which concluded that it is impressive but risky.

Still undecided is the fate of the proposed Lone Star detector, widely known as L\*, piloted by Samuel Ting of the Massachusetts Institute of Technology, who is at present the spokesman for the L3 detector at the successful Large Electron-Positron collider (LEP) at CERN in Geneva.

L\* was denied approval at this stage, but an amended proposal would be considered. The committee has made two particular suggestions. One is that, in what is essentially a multi-national collaboration, the proportion of US participants should be increased. This would reduce the financial vulnerability of the United States should one or more of the foreign participants back out.

SSC also asks that the top-level management of the project should be strengthened and expressed anxiety about the cost estimates for L\*, suggesting that the laboratory should independently review the figures to tell whether the costs are feasible.

One possibility now is that physicists from EMPACT/TEXAS will join forces with L\* and Ting says the two groups are discussing options such as this.

With or without the former competitors as team-members, Ting says he expects to report back to SSC in February for reconsideration. Formal proposals from Trilling's SDC and from L\*, if approved, are due in April 1992. Planners hope the SSC itself will be completed in 1999 at a cost of about \$8,250 million.

**Elizabeth Schaefer**

# Graphitic carbon copies

SIR—David Swinbanks, reporting from Tokyo (*Nature* 348, 186; 1990), represented both PAN (polyacrylonitrile)-based and pitch-based carbon fibres as having been invented in Japan. While this is clearly correct with regard to pitch-based fibre, I don't believe it to be true for the more important PAN-based fibres.

High-strength graphitic carbon fibre made by progressive oxidation and heat-treatment of polyacrylonitrile was developed by William Watt and his collaborators, L. N. Phillips and W. Johnson, working at the Royal Aircraft Establishment (RAE) in Farnborough, England, in the mid-1960s. According to Sir Alan Cottrell (at that time scientific adviser in the Cabinet Office), Watt was stimulated to this initiative by attending a Royal Society discussion on new materials in 1963 (A. H. Cottrell, *Proc. R. Soc. Lond. A* 319, 3–4; 1970). The RAE work was patented in 1964 and first published in 1966. In 1970, during a Royal Society discussion on strong fibrous solids, Watt gave a detailed account of the carbon fibres invented at the RAE (*Proc. R. Soc. Lond. A* 319, 5–15 1970;).

The new PAN-based fibre was manufactured in Britain by Courtaulds; US and Japanese manufacturers joined in too and, not for the first time and undoubtedly not for the last time in industrial history, the Japanese gradually cornered a large market share. It took a long time for carbon-fibre-reinforced polymer to be accepted in the marketplace, and there were major upsets on the way such as the Rolls-Royce experience with carbon-fibre-reinforced turbine fan blades. No doubt the Japanese manufacturers felt able to take a longer-term view than the British. None of this deprives Watt and his colleagues of the distinction of having invented and perfected one of the major new materials of the postwar period.

ROBERT W. CAHN

Department of Materials Science,  
University of Cambridge,  
Pembroke Street,  
Cambridge CB2 3QZ, UK

DAVID SWINBANKS REPLIES—PAN carbon fibre was invented by Akio Shindo at the Government Industrial Research Institute on Osaka and he published the work in 1961 (*Studies on Graphite Fibre*, Rep. No. 317, Gov. Ind. Res. Inst., Osaka, December 1961). He was awarded Japanese patents for the material in 1962 (patent number: Sho 37–4405) and 1963 (Sho 38–12375). British and US patents were also awarded to Tokai Denkyoku Seizo KK of Japan before the Royal Aircraft Establishment began its work.

William Watt and his colleagues referenced Shindo's earlier work in a

paper in *Nature* in 1987 (*Nature* 213, 690–691; 1987) and in subsequent correspondence in *Nature* they clarified that PAN carbon fibres were first made by Shindo and that their contribution was to increase the strength of the fibres by applying tension in the fibres during the early stages of preparation (*Nature* 220, 835; 1968). □

## Abstracting art

SIR—Much as I agree with the spirit of Rosner's criticisms (*Nature* 345, 108; 1990) of science as a product, I think that he misses a major reason for the spread of assertive sentence titles — the need for today's abstracting services (and today's researchers scanning *Current Contents* and the like) to compress as much information into as few words as possible so that the readers can decide whether a particular paper is worth looking at in full.

Consider Rosner's example of DNA. Studies showing that DNA is the genetic material and that it is not could both have the title, say, "A study of DNA and inheritance factors", or even just "DNA and the genetic material". Nor would a list of keywords be of help in distinguishing the two studies. Only by compressing their findings into a short (and, with skill, elegantly pithy) declarative title can the two studies distinguish themselves from one another for the scanners and abstracters of the world. This is a valid intermediate step in connecting the right people with the right research. The title is an identifier, not the work itself. Dissertation-type titles (all three or four lines of them) are not particularly appropriate for research articles, which meet a different need. As a compression, a title is bound to contain more flatly assertive statements than qualifiers ("DNA might be the genetic material"?). Besides, a paper's abstract and summary sections are themselves compressions of the research being reported.

But there is a danger that the drive to be succinct and informative will lead instead to sensationalist titles and the oversimplification, even outright misrepresentation, of the work. "DNA needs SEX." Now that I have your attention, I think that it is this danger that Rosner is really concerned about, and rightly so. As do Infante and Husazagh (*Nature* 346, 505; 1990), Rosner condemns the erosion of scholarship and the rise of simple-minded science brought on by the hubris of increasing technical sophistication gratifying popular expectations of what 'modern' science should be. These criticisms can be derived from Medawar's 1963 criticism ("Is the Scientific Paper a Fraud?" in *The Art of the Soluble*, Methuen, London) of the obsession of scientific reports with inductive reasoning, that mythical

theory-free route to scientific truth. To the detriment of all, induction is being discovered as for the first time, by a generation of naive, but well-funded (and thus arrogant), researchers (who also sit on funding boards) who think they need frame no hypotheses to obtain the answer. They are oblivious to the theory-dependency and conceptual ancestry of the research programmes they have been born into.

RICHARD T. O'GRADY

Department of Invertebrate Zoology,  
National Museum of Natural History,  
Smithsonian Institution  
Washington, DC 20560, USA

## How to get on

SIR—Selection committees often find it difficult to assess the research ability of those they interview. One way to overcome this problem would be to ask candidates for promotion to say what they consider to be their most important contribution to the literature and to illustrate its usefulness and originality by referring to papers from other laboratories that confirm their own work as well as papers that have used their observations as a step to further knowledge.

A. E. STUART

Airdshiel, Camus Cross,  
Isleornsay, Isle of Skye, UK

## Right to reply

SIR—The question "Why do simple organisms such as bacteria not develop the same complexity as other (organisms)?" (*Nature* 345, 707; 1990) is wrongly put: it reveals P. R. Sheldon as a eukaryotic chauvinist. We prokaryotes are not nearly as simple as we may seem to you. We can pack more biochemical versatility into a couple of cubic micrometres than can you in a couple of kilograms of liver, kidneys and other offal. We do not need hearts and lungs, as we breathe by diffusion. With propellers more efficient than yours, we can swim at speeds exceeding a thousand lengths a minute. To fly we don't need wings of bone, muscle and feather: we just get blown around effortlessly, and thereby we can get anywhere. And we can reproduce at rates many orders of magnitude higher than yours, producing offspring with considerably fewer initial contortions and no ultimate pain. Correspondingly we have evolved much, much faster. The trick to all this is, of course, miniaturization unrivalled by even the tiniest midge or the cleverest of human technologies.

So I would rephrase Sheldon's question thus: "Why do organisms such as bacteria not need to develop physical complexity?" The answer would then be obvious.

E. COLI

La Jolla, California, USA



## Elizabethan snobbery?

SIR—P. J. Smart's personal attack (*Nature* 348, 384; 1990) on Mr Stan Wood's motives in his sale of the earliest known reptile is reminiscent of the way in which Victorian snobbery retarded the advance of palaeontological science by decades by failing to take account of the work of aspiring researchers from the lower classes.

Smart's opinion of Wood is certainly not shared by those workers in site conservation, museums and vertebrate palaeontology who actually know anything about Wood's discoveries of novel forms and his infective enthusiasm, collaboration with researchers and conservationists, and financial grants to students at conferences.

Smart, by implication, would have approved if Wood had had a research grant from the Royal Museum of Scotland to pay his salary, workers' salary, rental of quarry, collaboration with researchers and museums, levy to local authority and

general operating overheads. So why is Smart upset that Wood has, as it were, carried out his research at his own risk, being paid only with hindsight after demonstrating his extraordinary success in finding the reptile and much else that could not have been foreseen?

MICHAEL A. TAYLOR

JOHN G. MARTIN

ARTHUR R. I. CRUICKSHANK

CHRISTOPHER J. COLLINS

Leicester, UK

SIR—In response to P. J. Smart, we wish to make the following points:

(1) At one time Mr Stan Wood was employed as a fossil collector, but when the grants supporting his work came to an end he was made redundant. Rather than accepting the extinction of his talent, he decided to try to make his living as a self-employed fossil collector. He passed through a period of considerable financial hardship and anxiety for his family, until

his discovery of sufficient new material to allow him to pay off his debts. To imply that he is avaricious is unjust.

(2) The price of "Lizzie" reflects not only its uniqueness, but also the expertise, time, logistical support and effort required for its discovery. The sum of money involved is trivial compared with the price of comparably important art works.

(3) Wood's professional openness and cooperation with researchers has consistently allowed proper studies to be made of his commercially available material. His work has been directly responsible for the inception of many research projects, including the completion of several PhDs.

(4) The price placed on this fossil material will help museum and research funding authorities to appreciate the value of our national collections (and the importance of their curation), and thus the relative value of properly funded fieldwork.

M. I. COATES

J. A. CLACK

K. A. JOYSEY

University Museum of Zoology,  
Downing Street, Cambridge CB2 3EJ, UK

## Leaving the lab

SIR—In a recent leading article, "Era of disappointment" (*Nature* 348, 375; 1990), you state that "It is curious that the first British prime minister with a technical qualification should have become so closely associated with the decline of basic science in Britain" and describe Mrs Thatcher as someone "expected to be sympathetic" to the cause of basic research.

My question is why should an ex-scientist be expected to have any more regard for science than a non-scientist? Ex-scientists arise in two main ways, either willingly or unwillingly. For examples of the former, one need think only of the significant numbers of students and postgraduate students who, at the end of a three-year stint, wish never to set foot in a laboratory again. These people would certainly not be "expected to be sympathetic" to science. Next, there, are those who, although they do not share the strong feelings of the first group, see no prospect of an acceptable career for themselves in science and leave to become accountants or tax inspectors (or politicians and prime ministers). This group spans most of the age range from the newest graduate to the established lecturer, and once they have made their move will presumably use any influence to improve the lot of their new profession rather than that of science.

Finally, there are those who leave science unwillingly, unable to find a job in their chosen field or unable to fund their research. These people are often further displeased by the uncaring attitude to their plight of many established academics whose sole concern is their own next

grant. This last group, having doubtless invested several years in science before being cast adrift, have no reason to regard the scientific establishment with any fondness.

So, to answer my own question, ex-scientists are, if anything, likely to be less sympathetic to the furthering of basic science than non-scientists. Certainly no sympathy should be expected on the basis of their past associations. This last applies particularly to all politicians on any subject — just ask former prime minister Mrs Thatcher.

ALLWYN HART

15 Laurel Close,  
Potters' Green, Coventry CV2 2NH, UK

## Sentimental journey

SIR—The leading article "Era of disappointment" makes an observation by the US historian Richard Hofstadter in the 1955 book, *The Age of Reform*, seem very relevant. In the introduction, he noted:

While it is always both feasible and desirable to formulate ideal programs of reform, it is asking too much to expect that history will move, so to speak, in a straight line to realize them. Liberal intellectuals, who have rather well-rationalized systems of political beliefs, tend to expect that the masses of people, whose actions at certain moments in history coincide with some of these beliefs, will share their other convictions as a matter of logic and principle. Intellectuals, moreover, suffer from a sense of isolation which they usually seek to surmount by finding ways of getting into a rapport with the people, and they readily

succumb to a tendency to sentimentalize the folk. Hence they periodically exaggerate the measure of agreement that exists between movements of popular reform and the considered principles of political liberalism (p. 19).

This seems appropriate to Margaret Thatcher as well as to the leader writer.

DAVID L. WAGGER

Massachusetts Institute of Technology,  
Department of Chemical Engineering,  
25 Ames Street, Bldg 66-553,  
Cambridge, Massachusetts 02139, USA

## Weighty argument

SIR—Let's temper the bicentennial celebration of the metric system (*Nature* 348, 105; 1990) with a few sobering literary facts. Had the metric revolution come sooner or been sweeping, we would be heirs to a cultural wasteland. Pound for pound (kilogram for kilogram?), metric units are as cold as bone. For the bards, the British Imperial System was the measure of verse. Shakespeare's Shylock never demanded his 454 grams of flesh, and Robert Frost never had "kilometres to go before I sleep". How far would Erskine Caldwell have gone with *God's Little Hectare*, or Jules Verne with *96,450 Kilometres Under the Sea*? Clearly, in literature an ounce of metric prevention was worth a pound of cure. Metric units have made one inroad into day-to-day language: kilo is the measure and talk of the drug trade.

LEONARD KRISHTALKA

Department of Vertebrate Paleontology,  
Carnegie Museum of Natural History,  
4400 Forbes Avenue, Pittsburgh,  
Pennsylvania 15213 USA

# Towards a paradigm shift in biology

The steady conversion of new techniques into purchasable kits and the accumulation of nucleotide sequence data in the electronic data banks leads one practitioner to cry, "Molecular biology is dead — Long live molecular biology!".

THERE is a malaise in biology. The growing excitement about the genome project is marred by a worry that something is wrong — a tension in the minds of many biologists reflected in the frequent declaration that sequencing is boring. And yet everyone is sequencing. What can be happening? Our paradigm is changing.

Molecular biology, from which has sprung the attitude that the best approach is to identify a relevant region of DNA, a gene, and then to clone and sequence it before proceeding, is now the underpinning of all biological science. Biology has been transformed by the ability to make genes and then the gene products to order. Developmental biology now looks first for a gene to specify a form in the embryo. Cellular biology looks to the gene to specify a structural element. And medicine looks to genes to yield the body's proteins or to trace causes for illnesses. Evolutionary questions — from the origin of life to the speciation of birds — are all traced by patterns on DNA molecules. Ecology characterizes natural populations by amplifying their DNA. The social habits of lions, the wanderings of turtles and the migrations of human populations leave patterns on their DNA. Legal issues of life or death can turn on DNA fingerprints.

And now the genome project contemplates working out the complete DNA pattern and listing every one of the genes that characterize all of the model species that biologists study — ourselves even included.

At the same time, all of these experimental processes — cloning, amplifying and sequencing DNA — have become cook-book techniques. One looks up a recipe in the Maniatis book, or sometimes simply buys a kit and follows the instructions in the inserted instructional leaflet. Scientists write letters bemoaning the fact that students no longer understand how their experiments really work. What has been the point of their education?

The questions of science always lie in what is not yet known. Although our techniques determine what questions we can study, they are not themselves the goal. The march of science devises ever newer and more powerful techniques. Widely used techniques begin as breakthroughs in a single laboratory, move to being used by many researchers, then by technicians,

then to being taught in undergraduate courses and then to being supplied as purchased services — or, in their turn, superseded.

Fifteen years ago, nobody could work out DNA sequences, today every molecular scientist does so and, five years from now, it will all be purchased from an outside supplier. Just this happened with restriction enzymes. In 1970, each of my graduate students had to make restriction enzymes in order to work with DNA molecules; by 1976 the enzymes were all purchased and today no graduate student knows how to make them. Once one had to synthesize triphosphates to do experiments; still earlier, of course, one blew one's own glassware.

Yet in the current paradigm, the attack on the problems of biology is viewed as being solely experimental. The 'correct' approach is to identify a gene by some direct experimental procedure — determined by some property of its product or otherwise related to its phenotype — to clone it, to sequence it, to make its product and to continue to work experimentally so as to seek an understanding of its function.

The new paradigm, now emerging, is that all the 'genes' will be known (in the sense of being resident in databases available electronically), and that the starting point of a biological investigation will be theoretical. An individual scientist will begin with a theoretical conjecture, only then turning to experiment to follow or test that hypothesis. The actual biology will continue to be done as "small science" — depending on individual insight and inspiration to produce new knowledge — but the reagents that the scientist uses will include a knowledge of the primary sequence of the organism, together with a list of all previous deductions from that sequence.

How quickly will this happen? It is happening today: the databases now contain enough information to affect the interpretations of almost every sequence. If a new sequence has no match in the databases as they are, a week later a still newer sequence will match it. For 15 years, the DNA databases have grown by 60 per cent a year, a factor of ten every five years. The human genome project will continue and accelerate this rate of increase. Thus I expect that sequence data for all of the model organisms and half of

the total knowledge of the human organism will be available in five to seven years, and all of it by the end of the decade.

To use this flood of knowledge, which will pour across the computer networks of the world, biologists not only must become computer-literate, but also change their approach to the problem of understanding life.

The next tenfold increase in the amount of information in the databases will divide the world into haves and have-nots, unless each of us connects to that information and learns how to sift through it for the parts we need. This is not more difficult than knowing how to access the scientific literature as it is at present, for even that skill involves more than a traditional reading of the printed page, but today involves a search by computer.

We must hook our individual computers into the worldwide network that gives us access to daily changes in the database and also makes immediate our communications with each other. The programs that display and analyse the material for us must be improved — and we must learn how to use them more effectively. Like the purchased kits, they will make our life easier, but also like the kits, we must understand enough of how they work to use them effectively.

The view that the genome project is breaking the rice bowl of the individual biologist confuses the pattern of experiments done today with the essential questions of the science. Many of those who complain about the genome project are really manifesting fears of technological unemployment. Their hard-won PhDs seem suddenly to be valueless because they think of themselves as being trained to a single marketable skill, for a particular way of doing experiments. But this is not the meaning of their education. Their doctorates should be testimonials that they had solved a novel problem, and in so doing had learned the general ability to find whatever new or old techniques were needed; a skill that transcends any particular problem.

**Walter Gilbert**

*Walter Gilbert is Carl M. Loeb university professor in the Department of Cellular and Developmental Biology at Harvard University, in the Biological Laboratories, 16 Divinity Avenue, Cambridge, Massachusetts 02138 USA.*



*Tim Hunt*

The diagram illustrates the regulatory mechanism of the cell cycle. It shows a cycle where an inactive complex of Cyclin and cdc2 is activated by the addition of a phosphate group (P) via a tyrosine kinase. The active complex, labeled 'Active', is then inactivated by the removal of the phosphate group via a tyrosine phosphatase. Finally, the active complex is destroyed, leading to 'Cyclin destruction' and the release of free cdc2, which remains inactive. The free cdc2 then combines with new Cyclin to restart the cycle.

```
graph TD
    Inactive1[Inactive Cyclin cdc2] -- "Tyrosine kinase" --> Active[Active Cyclin cdc2 P]
    Active -- "Tyrosine phosphatase" --> Inactive2[Inactive Cyclin cdc2]
    Inactive2 -- "Cyclin destruction" --> cdc2_free[cdc2 Inactive]
    cdc2_free -- "Cyclin" --> Inactive1
```

that phosphorylation of p34<sup>cdc2</sup> on tyrosine 15 keeps its kinase activity turned off, and activation of p34<sup>cdc2</sup> is associated with dephosphorylation at tyrosine 15. When frog eggs are fertilized and return to interphase, cyclin is destroyed and cdc2 kinase activity rapidly lost, but tyrosine 15 is not immediately rephosphorylated. Solomon *et al.* found that phosphorylation of p34<sup>cdc2</sup> on tyrosine 15 requires cyclin — but why should activation of the kinase be preceded by a known inhibitory modifica-

ubiquitinated lysine  
|  
RHLOLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLPKKT  
RHILLAIRNDEELNKLMANTTIADGGVLPNINPMLLPSKSKKT

So much for activation of cdc2 kinase. What is responsible for the rapid, specific and exquisitely timed proteolysis of cyclin that turns the kinase off? The specificity of the system is remarkable, both in terms of its targets and its timing. Cyclins seem to be the sole substrate for the protease concerned, and they are perfectly stable in interphase extracts. In mitotic extracts about to enter anaphase, they are destroyed in seconds (the notable exception to this rule being frog eggs, where the

The first real clues to the destruction mechanism came from a deletion analysis of the cyclin molecule. The N terminus of both A- and B-type cyclins is dispensable for cdc2 kinase activation<sup>[4]</sup>, and the sequence of the first 100–150 residues is highly variable, even in the closely related B1 and B2 cyclins or between the B-type cyclins of two species of sea urchin. But embedded in this variable leader is a small conserved island, the destruction box (see Fig. 2), which Glotzer *et al.*<sup>2</sup> now show to be necessary and sufficient for programmed mitotic proteolysis of proteins containing it. The box comes in two parts, the partially conserved motif Arg-X-X-Leu-X-X-Ile-X-Asn (RXXLXXIXN in the single-letter amino-acid code) being followed by a lysine-rich stretch. Most

1. Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D. & Hunt, T. *Cell* **33**, 389–396 (1983).
2. Glotzer, M., Murray, A. W. & Kirschner, M. W. *Nature* **349**, 132–138 (1991).
3. Draetta, G. *et al.* *Cell* **56**, 829–838 (1989).
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5. Gould, K. L. & Nurse, P. *Nature* **342**, 39–45 (1989).
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9. Luca, F. & Ruderman, J. V. *J. Cell Biol.* **109**, 1895–1909 (1989).
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12. Jentsch, S., Seufert, W., Sommer, T. & Reins, H.-A. *Trends biochem. Sci.* **15**, 195–198 (1990).
13. Roy, L. M. *et al.* *Cell* **61**, 825–831 (1990).
14. Busch, H. & Goldkorn, I. L. *Molec. cell. Biochem.* **40**, 173–187 (1981).



strikingly, mutation of the highly conserved arginine (Arg 42→Cys (C)) in the destruction motif stabilizes cyclin.

Glötzer *et al.*<sup>2</sup> developed a clever assay system, in which destruction was locked permanently on by addition of non-biodegradable sea-urchin cyclin made in bacteria, and demonstrate that the cyclin is being polyubiquitinated<sup>11</sup>. The obvious experiment of inhibiting the ubiquitin system with a specific inhibitor was impossible, though Glötzer *et al.* were able to show that the flux through the ubiquitinated intermediates was adequate to account for the overall rate of destruction. The case is persuasive, but not yet closed.

These experiments only begin to explain how cyclin destruction is regulated. It is simplest to imagine a cyclin-specific ubiquitin ligase (see ref. 12 for a review of these enzymes) that recognizes the destruction box and is regulated by cell cycle-specific phosphorylation. The ligase would be activated (probably indirectly, to account for the lag) by cdc2 kinase. Metaphase arrest in eggs would be brought about by *c-mos* kinase, which would keep the hypothetical ligase turned off. (This might also explain the trans-

forming effects of *c-mos*, if hypothetical G1→S cyclins were stabilized in a similar way.) Cyclin is reportedly a substrate for *c-mos*<sup>13</sup>, which might inhibit the attachment of ubiquitin. The two extreme hypotheses — regulation of the enzyme or regulation of the substrate — are not mutually exclusive; and if the activation of cdc2 kinase is any guide, a process so critical in the life of a cell as the transition from metaphase to anaphase may be regulated in a sophisticated way.

One of the first places where ubiquitin was recognized was in a variant histone H2A<sup>14</sup>. It is intriguing that close to lysine 119, which becomes covalently modified by ubiquitin, there is a sequence with some resemblance to the cyclin destruction box (Fig. 2). Are RAALGNISN and variants of this sequence components of the recognition motif for ubiquitin ligases? Why are some proteins multiubiquitinated and proteolysed, whereas others (for example histone H2A) are reversibly monoubiquitinated? There is indeed much to be discovered. □

Tim Hunt is at the ICRF Clare Hall Laboratories, South Mimms, Herts EN6 3LD, UK.

MATERIALS SCIENCE

## When size does matter

Philip Ball

BETWEEN theories of the behaviour of matter on the atomic and the macroscopic scales is a gap that has never quite closed. Similarly, descriptions of bulk phase behaviour seldom survive intact when extended into the interfacial region between phases. But a recent meeting of the Materials Research Society\* made clear that the nanoscale structure (containing a numerable quantity of atoms) and the interface pose challenges that carry rich rewards for theorist and experimentalist alike.

The two are not, of course, independent concerns, as nanostructures are often largely surface anyway. Their importance to catalysis is therefore a natural consequence. John Bradley (Exxon, Annandale) described the problems in and some solutions to creating colloidal suspensions of transition-metal clusters in the nanometre size range — systems in which one might hope to combine some of the advantages of both homogeneous catalysts (such as solvated transition-metal complexes) and heterogeneous ones (such as bulk metal surfaces).

The particles must be prevented from aggregating and precipitating, but too efficacious a protective coating will also be detrimental to their catalytic activity. The answer is to form the particles in solution

in the presence of polymers that bind only weakly to their surfaces: for instance, palladium clusters a few nanometres in diameter can be prepared in the presence of polyvinyl pyrrolidone. A clue to their catalytic properties can be gained from the adsorption of carbon monoxide. To smaller particles (around 1 nm diameter), CO binds 'end on' — as in the molecular carbonyl Pd(CO)<sub>n</sub> — whereas on larger clusters (around 7 nm), which require a different stabilizing polymer, one finds bridging of CO across two metal atoms, as on the bulk solid.

A variant on this approach to colloidal metal sols is to prepare the particles inside inverse micelles (Jess Wilcoxon, Sandia National Laboratories). These are micellar structures — spherical agglomerations of amphiphilic molecules — in which the hydrophilic heads point inwards rather than outwards, exposing the hydrophobic tails to the solvent. Naturally, they are formed in hydrophobic organic solvents. Transition-metal salts, which tend to be hydrophilic, can be encapsulated inside inverse micelles, whereupon reduction followed by homogeneous nucleation and growth leads to metal particles stabilized by a coating of amphiphiles.

Immobilizing small metal clusters on a supporting surface may make for a catalyst that is more easily recycled. But depositing the clusters directly from a

vapour phase can be disruptive. A gentler method (John Weaver, University of Minnesota) is to allow the clusters to aggregate from the metal vapour on a soft buffer layer of an inert gas such as xenon deposited on the support. Diffusion of metal atoms over the xenon layer is facile, and control of the cluster size is also accomplished easily. As xenon tends to wet metals such as silver and gold, clusters of these metals will become enveloped by the buffer, then slowly sink down to the substrate. Evaporation of the xenon exposes the supported clusters. This kid-gloved technique can produce defect-free, well-crystallized particles.

Richard Masel (University of Illinois) suggested the possibility of even greater control over the properties of supported metal clusters, by selecting the specific crystallographic orientation of the exposed facets. Certain crystal faces are more reactive than others, a factor that may far outweigh the surface-area effect on catalytic rates. As the orientation of facets is determined by their relative surface energies, control of surface energy by adsorption of gases may promote one face over the others. For example, Masel finds that annealing of platinum microcrystallites in hydrogen produces cubic particles with (100) faces exposed, whereas a hydrocarbon atmosphere gives cubooctahedra. Likewise, CO promotes (210) faces, and nitrogen, which reduces the energy of step formation, produces spherical particles.

Kenneth Suslick (University of Illinois) demonstrated impressive control of metal particle morphology using ultrasound, which corresponds to acoustic frequencies of kilohertz to megahertz. The intense compression and expansion produced by these waves can create local heating of around 5,000 K and pressures of up to 1,000 atmospheres, along with cooling rates of about 10<sup>6</sup> K s<sup>-1</sup>. Glassy metal phases ('met-glasses') of very high surface area, and thus catalytic activity, can result from ultrasonication of solutions of transition-metal complexes such as Fe(CO)<sub>5</sub>. The high-velocity collisions induced between particles can cause local melting, leading to particle aggregation. Surface chemistry may also be influenced by these intense conditions. Powdered nickel taken 'off the shelf' showed a thousand-fold increase in hydrogenation rate after irradiation with ultrasound, a consequence of the almost complete removal of the surface oxide layer.

Don Eigler (IBM Almaden) confessed to a more brutal, though also more dexterous, approach to surface reactions. He has attempted to build molecules by pushing their components together in a shotgun marriage that uses the tip of a scanning tunnelling microscope as the firearm. Xenon atoms will yield to the persuasion quite readily (see *Nature* 344, 524–526;

\*Fall Meeting of the Materials Research Society, Boston, 26–30 November 1990.

1990), and indeed Eigler has now formed them into closed stockades — perhaps the world's smallest and most inert beaker. But adsorbed oxygen atoms and CO molecules, from which Eigler hopes to create CO<sub>2</sub>, are less amenable: oxygen refuses to budge at all, whereas CO will do scarcely more. Even if a more slippery surface can be found, there will be the activation energy barrier to be surmounted, and then the heat of reaction might send the product molecules skidding out of sight.

The surface chemistry of more complex molecules, such as organic polymers, raises a new set of issues. For instance, at what point in the polymer chain do they become attached to the substrate, and what steric effects are to be expected from these bulky species in the vicinity of the surface? At low coverages chain-like polymers will maximize surface interactions by lying flat, but higher coverages produce a hairy surface with each chain attached by several contacts towards one end (Pierre de Gennes, Collège de France). The dynamics of incoming polymers can be described in terms of motion on a self-similar grid, dominated by the inner (most dense) portion.

For George Whitesides (Harvard University), the surface was no idealized solid substrate but the protein coat of a virus. Attaching polymers to such a surface might provide a means of blocking the highly specific binding of the virus to a cell surface. The influenza virus, for example, recognizes sialic acid groups on a cell membrane, so that a polymer comprised of monomers that contain sialic acid might act as a viral inhibitor. Whitesides suggested that such artificial inhibitors might be orders of magnitude more effective than natural ones.

Whitesides has succeeded in depositing protein monolayers on gold to aid recognition studies of this sort. Troy Wilson (University of California at Berkeley) reported the formation of similar monolayers, containing amino-acid groups, that should also provide useful substrates for molecular recognition. These films are made particularly robust by incorporating acetylene groups part of the way along the aliphatic chains — irradiation then promotes cross-linking polymerization to give a film that can be transferred more or less intact onto almost any hydrophobic surface. The electronic and spectroscopic properties of these films hint at a potential for organic semiconductor applications.

Optoelectronics lies more explicitly at the heart of the studies by Robert Birge and colleagues (Syracuse University) of a genuinely natural material, bacteriorhodopsin. This protein is used for light harvesting by *Halobacterium halobium*, which grows in salt marshes. Oriented bacteriorhodopsin in a polymer matrix provides a versatile component for optical computing applications as a result of its

ability to switch photochemically between two states that absorb at different wavelengths, with a switching time of less than five picoseconds. Birge *et al.* have used bacteriorhodopsin in computer architectures as a light modulator (an applied voltage or a second light beam controls the absorption) and as the basis of two memory devices: a random-access memory with access times of a few nanoseconds and an associative memory device (for which an

VISUAL PERCEPTION

## Grasping the essentials

Alan Cowey

AT the turn of the century, the Swiss psychologist Claparède did something that present-day medical ethics committees would almost certainly forbid<sup>1</sup>. Shaking hands with a patient suffering from Korsakoff's psychosis, a profound disorder of memory that prevents the sufferer from remembering recent experiences, he pricked her hand with a pin concealed between his fingers. As usual at their next meeting she failed to recognize him, but declined to shake hands, insisting that it might be unpleasant although she was at a loss to explain why.

Claparède had demonstrated the phenomenon of covert awareness, the paradoxical inconsistency between overt behaviour and memory or between what Claparède called actions and the patient's cognitive inability to recognize or recall their antecedents. He noted other pathological examples and applied the distinction to phenomena as different as *déjà vu* (an overpowering feeling of familiarity without recognition) and post-hypnotic states where the subject may produce word associations learned under hypnosis but without any recollection of having learned them (recall without attendant familiarity). A report by Goodale *et al.* on page 154 of this issue<sup>2</sup> provides a novel example of a similar dissociation between perceptual awareness and action.

Dissociations of this kind remained as scientific curiosities until the 1970s, despite the fact that John MacCurdy, lecturer in psychopathology at Cambridge, had devised a method of studying them objectively and quantitatively in the 1920s. MacCurdy<sup>3</sup> presented an amnesic patient with his (MacCurdy's) name and address, which were promptly forgotten. Later he presented the patient with a list of first names, street names and numbers, and asked the patient to guess which of them were his. "To my surprise, the guesses were nearly as accurate as would be the conscious memory of such data of normal subjects." Although he is rarely given credit for it, MacCurdy had invented the now widely used method of forced-choice guessing to uncover covert

input triggers a search for related but not necessarily identical patterns).

Birge's theme was echoed in several diverse presentations on the mechanical properties of cellular materials such as wood and fibrous ones such as horn and grass: one can do far worse than look to the natural world for ingenious answers to problems in materials design. □

Philip Ball is an assistant editor of Nature.

knowledge. In the past 15 years its use has revealed an astonishing degree of covert or implicit awareness in normal subjects and in brain-damaged patients with respect to such things as memory, facial recognition, and sensory detection and discrimination (see ref. 4 for review).

The new discovery by Goodale *et al.*<sup>2</sup> concerns a young woman, D.F., who became visually agnostic following carbon monoxide poisoning. She is unable to recognize objects or shapes by sight despite having full visual fields and full understanding of the nature of the tasks. So severe is her agnosia that she cannot tell whether a line is vertical or horizontal, or whether a square and rectangle are the same or different. Of the several varieties of visual agnosia, D.F. has apperceptive or pseudoagnosia. The classification is important because this extreme form of visual agnosia is usually thought to indicate a disturbance of visual processing at a very early stage of visual cortical sensory representation, perhaps at the level of what David Marr<sup>5</sup> called the two-and-a-half dimensional sketch. If this view is correct the patient should not show covert recognition because nothing can be recognized that is not adequately represented in the first place. Goodale *et al.* have uncovered covert recognition in D.F. by using a novel procedure and have thereby demonstrated that an effective representation of shape, size and orientation must exist at some level of the visual pathways.

How did they manage it? Although D.F. could not turn a hand-held card until its orientation matched that of a slot in front of her, she could 'post' the card skilfully through the slot. Furthermore, when asked to reach out for objects that she could neither identify nor tell apart, her hand position was appropriately adjusted for accurate grasping. Finally, although she could neither state whether two simple shapes were the same or different, nor indicate their length and breadth with her thumb and index finger, she nevertheless positioned her fingers normally when asked to pick up those objects.



In other words, D.F. must be able to perceive objects and their orientation in order to grasp or approach them despite failing to recognize or discriminate them. Her extreme apperceptive agnosia is therefore not caused by an inability to create what are often called neural images of her visual surroundings.

Why are findings such as this important? Patient D.F. demonstrates with arresting clarity that not all complex computations involved in the neural representation of shape, size and orientation are accessible to conscious judgements of these object qualities. It is as if conscious awareness operated on a need-to-know basis, and that many neural events such as those governing prehension can occur without awareness in parallel with others of a similar nature that lead to conscious awareness. As Goodale *et al.* themselves suggest, the initial neural processing underlying shape perception may be unitary, and different 'parallel' channels may then mediate our ability to recognize the object and to reach for it. Destroy the channel concerned with recognition and one is left with symptoms like those of D.F. In this sense D.F. shows the dissociation between action and cognition that so struck Claparède and which, long after its discovery, is now seen as one of the strongest pieces of evidence for the modular operation of the brain.

Not surprisingly, the new report prompts further questions. For example, accurate prehension has been used as evidence that babies too young to respond in other ways can perceive shape. The natural supposition is that their shape perception is also conscious and cognitive, like ours. Patient D.F. shows that it may not be. The remarkable accuracy with which she reaches for objects and aligns them suggests that she, and perhaps other severely agnostic patients, could be trained to recognize shape and orientation by paying attention to what her hand is doing. But does she know whether her hand is vertically or horizontally oriented? Could patients with other dissociations of perception and awareness reveal covert recognition by their voluntary movements? For example, patients with 'blindsight' who can point to targets they fail to 'see', have defied all attempts to demonstrate genuine shape discrimination by forced-choice guessing. Perhaps it is time to examine their grasp as well as their reach. □

Alan Cowey is in the Department of Experimental Psychology, University of Oxford, South Parks Road, Oxford OX1 3UD, UK.

# Starquakes and $\gamma$ -ray bursts

Paul C. Joss

SINCE their discovery in 1973, cosmic  $\gamma$ -ray bursts have become the most persistently enigmatic of astronomical phenomena. Suggested mechanisms have ranged from antimatter micrometeors impinging on the heliopause to vast explosions occurring shortly after the Big Bang. Although some of the more exotic ideas have been ruled out, we have remained

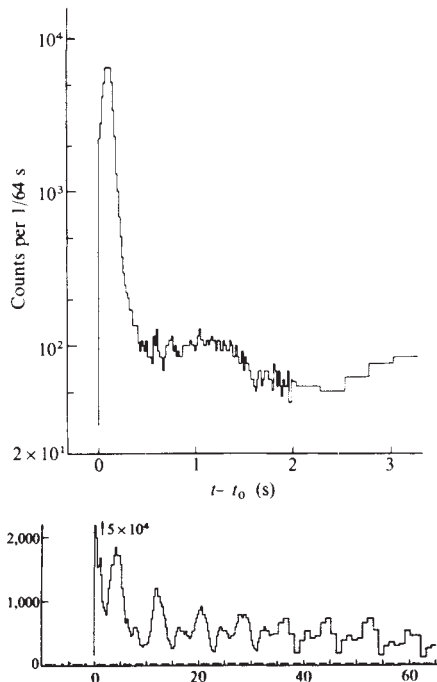


Fig. 1 Intensity profile of the  $\gamma$ -ray burst of 5 March, 1979. This notably smooth and regular burst was also the most intense that has so far been seen. The lower panel shows periodic pulsations in the source intensity during the burst's decline. (Adapted from ref. 9.)

woefully ignorant of the underlying cause of these events. Now Blaes, Blandford, Madau, and Koonin, in *The Astrophysical Journal*<sup>1</sup>, reexamine the conjecture that  $\gamma$ -ray bursts result from seismic events in the solidified crusts of old neutron stars in our Galaxy and demonstrate that this type of mechanism may, after all, account for the phenomenon. If this is, indeed, the correct mechanism, then a united picture of the phenomenology of galactic neutron stars may now be within reach.

Neutron stars have long been prime candidates as the sources of  $\gamma$ -ray bursts. The most persuasive line of evidence in this direction has been the detection of pulsed modulation, with pulse periods of the order of seconds (compatible with the rotation period of a neutron star), in the burst profiles of some events (Fig. 1). The discovery in recent years of sharp features in the spectra of some bursts, at photon energies of tens of kiloelectronvolts, has lent further credence to the view that

neutron stars are the sites of the bursts. Such features can be understood as cyclotron-radiation lines produced in the vicinity of a neutron star with a surface magnetic induction of around  $10^{12}$  gauss. Magnetic fields of this strength are modest for neutron stars and are routinely inferred to be present in radio pulsars. There are no known viable alternative explanations for these spectral features.

Most of the early theoretical work on neutron stars as the sites of  $\gamma$ -ray bursts concentrated on two specific mechanisms: collisions between neutron stars and other small bodies, such as comets or meteors (as first suggested by Harwit and Salpeter<sup>2</sup>), and thermonuclear flashes in the surface layers of accreting neutron stars<sup>3,4</sup>. Both of these proposed mechanisms, however, have been found to suffer from serious difficulties. The drawbacks in collision models are the inevitable tidal disruption of the infalling body and, most importantly, the rapid quenching of the  $\gamma$ -ray emission owing to the expansion and cooling of the plasma released in the impact. Similarly, thermonuclear flash models must deal with the overwhelming tendency of the individual photon energies to degrade into the X-ray range by the time the flash energy has propagated to the neutron-star photosphere. This situation is analogous to what happens in ordinary stars like the Sun: although the bulk of the solar interior is bathed in X-rays with energies in the kiloelectron-volt range, the solar surface must be substantially cooler (for the interior thermal energy to propagate outward) and radiates photons with typical energies of less than 1 electronvolt.

The idea that starquakes on neutron stars might cause  $\gamma$ -ray bursts was first suggested by Pacini and Ruderman<sup>5</sup>, Tsygan<sup>6</sup>, and Fabian, Icke and Pringle<sup>7</sup>. The most serious drawback of this picture has been the lack of any convincing energy source for the starquake. Now Blaes *et al.* present a series of elegant calculations to show that the gradual accretion of material (at a rate of around  $10^{10}$  g s<sup>-1</sup>) from the interstellar medium by an isolated neutron star, followed by the nuclear processing of this material via pycnonuclear (density-induced fusion) reactions, can result in the development of substantial density inversions (with density contrasts of around 5 per cent) some hundreds of metres beneath the surface of the star.

Although the details of their calculations are still subject to a number of uncertainties, mainly in the relevant nuclear physics, the authors have nevertheless built a convincing case that such inversions are likely to occur. They go on to

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## Enzyme therapy

THE lung secretions of cystic-fibrosis sufferers with respiratory bacterial infections contain large amounts of neutrophil-derived DNA; such secretions are consequently much more viscous than normal. S. Shak *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **87**, 9188–9192; 1990) have now cloned and expressed human recombinant DNase I, and show that it can dramatically reduce the viscosity of the thickened sputum *in vitro* but has no effect on the mucus of unaffected individuals. Taken in aerosol form, the pure human DNase I may prove to be a safe and effective way of aiding the daily clearance of sputum in patients suffering from cystic fibrosis. Preliminary clinical studies are said to be encouraging.

## Bang on

WE may be living in an unstable Universe that could explode because of a chance collision between cosmic rays, J. Ellis, A. Linde and M. Sher suggest. They make this rather extraordinary claim in a paper in *Physics Letters* (**B252**, 203–211; 1990) in which they try to pin down the mass of two of the most important as-yet-undiscovered fundamental particles, the top quark and the Higgs boson. Although the authors come to no conclusions about the masses of these particles, they do put forward ideas about the consequences of certain plausible values. In particular, if the top quark's mass is greater than some critical value determined by the mass of the Higgs boson, the Universe is not stable but rather is trapped in a long-lived unstable state out of which it could be precipitated by some violent event. Cosmic rays could provide the necessary trigger, and whether they have failed yet to do so because the Universe is insufficiently unstable, or because of sheer chance, is the big question.

## Blooming genes

MOLECULAR genetic analysis of flower development continues apace with the report from E.S. Coen *et al.* (*Cell* **63**, 1311–1322; 1990) of the cloning of a snapdragon gene that seems to be a master switch in the initial specification of flower-forming meristems. In plants with mutations in the gene (known as *floricaula*, or *flo* for short), vegetative meristems still give rise to inflorescence meristems, but these fail to produce flower-forming meristems and instead generate additional indeterminate shoots. Examination of the sequence of the *flo* protein product has not yet revealed any notable similarities to known protein sequences, though near the N terminus is a region strikingly rich in proline residues. The new work follows closely on the cloning of two 'flower homeotic' genes, which specify organs within a flower — at some level, presumably, genes of this class must come under *flo*'s control.

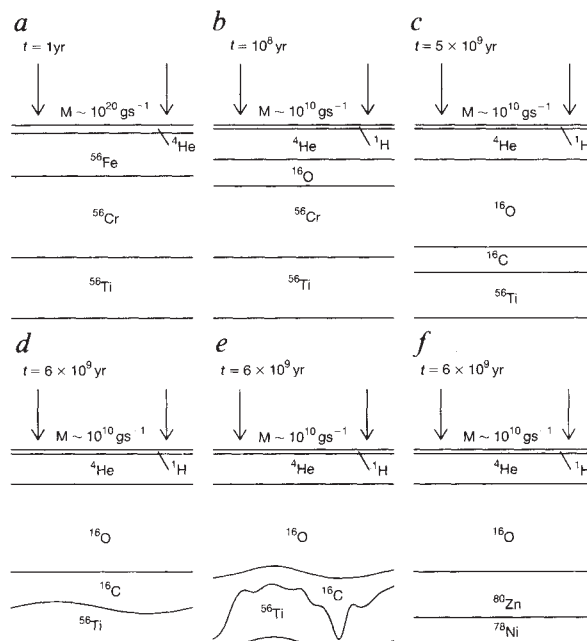


FIG. 2 Schematic sketch of one possible history of the surface layers of an isolated neutron star (from Blaes *et al.*<sup>1</sup>). Following its formation in a supernova event at time  $t = 0$  and a brief subsequent episode of rapid accretion from the surrounding supernova remnant, the star settles down to a much lower rate of accretion from the interstellar medium. The chemical composition of the surface layers is determined by the rates of various pycnonuclear (density-induced fusion) reactions as the accreted material is gradually compressed to ever higher densities. An appreciable density inversion eventually forms at the interface between carbon and titanium (note that in these layers the nuclei form a body-centred-cubic lattice in a sea of relativistic degenerate electrons). The interface becomes unstable at  $t = 6 \times 10^9$  yr, resulting in overturn and the release of gravitational and nuclear energy. This energy propagates to the stellar surface as seismic waves and may then lead to the emission of  $\gamma$ -radiation from the magnetosphere (see text).

demonstrate that once an inversion becomes sufficiently strong, the interface becomes unstable to an elastic Rayleigh–Taylor mode (Fig. 2). Finally, they argue that only a small fraction (about  $10^{-3}$ ) of the neutron star's surface 'breaks' in any given event, resulting in a localized overturn of the accreted matter and the release of  $10^{38}$ – $10^{41}$  ergs of gravitational and (mostly) nuclear energy.

This is just about the right amount of energy per event if  $\gamma$ -ray bursts originate on neutron stars in our own Galaxy. Moreover, variations in the size and shape of the quaking region, together with variations in the effects of projection and neutron-star rotation, may account for many of the complexities and case-to-case differences in the profiles of  $\gamma$ -ray bursts (in sharp contrast with the phenomenon of cosmic X-ray bursts, whose burst profiles are generally much smoother and more regular). Most importantly, the continual accretion provides a replenishable source of fuel for driving at least  $10^6$  bursts during the lifetime of every isolated neutron star in the Galaxy, as required for consistency with the frequency with which bursts are observed.

The energy release occurs so deeply beneath the neutron star's surface that very little of the energy is propagated to the photosphere via electron conduction or other thermal processes, nor do the surface layers expand appreciably. In a preceding paper<sup>8</sup>, some of the authors argued that the energy is conveyed to the surface mainly by seismic processes. There, the disturbed neutron star's magnetic field couples the released energy to the magnetosphere by generating intense, relativistic Alfvén waves. The final piece of the picture, namely, the conversion of these waves into relativistic particle motions and thence to  $\gamma$ -rays, constitutes an immensely difficult problem and has not yet been studied with any degree of rigour. Nevertheless, Blaes *et al.*<sup>8</sup> were able to present various plausibility arguments that the available energy can be efficiently converted to  $\gamma$  radiation on a timescale consistent with  $\gamma$ -ray burst phenomenology.

If the basic picture painted by Blaes *et al.*<sup>1,8</sup> turns out to be correct, we may be witnessing the emergence of a unified theoretical framework for understanding the phenomena of  $\gamma$ -ray burst

sources, X-ray burst sources, X-ray pulsars and radio pulsars. According to this view,  $\gamma$ -ray bursters are mostly isolated neutron stars that are accreting matter very slowly from the interstellar medium. Most X-ray bursters are neutron stars that are accreting matter at higher rates (perhaps  $10^{16}$  g s<sup>-1</sup>) from low-mass binary companion stars and that burn their nuclear fuel in discrete flashes at relatively shallow depths (tens of metres) beneath the neutron-star photosphere. The transfer of the thermonuclear energy released in a flash to the neutron-star photosphere via thermal processes leads to the emission of an X-ray burst.

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Most X-ray pulsars are neutron stars accreting matter at still higher rates (around  $10^{18}$  g s<sup>-1</sup>) from more massive binary companions; the high accretion rates combined with strong surface magnetic fields inhibit thermonuclear flashes and prevent the emission of X-ray bursts, while the fields funnel the accretion flow onto magnetic polar regions, giving rise to pulsations as the star rotates. Finally, any of these neutron stars could instead be a radio pulsar if the surface magnetic fields

were sufficiently strong (and misaligned with the rotation axis) and/or the stellar rotation period were sufficiently short to turn on the pulsar emission mechanism; the radiation and particulate wind generated by the radio pulsar would then quench any accretion and would thereby prevent the star from exhibiting other  $\gamma$ -ray or X-ray phenomena. □

Paul C. Joss is in the Department of Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

## EVOLUTIONARY BIOLOGY

# Borrowed sexual ornaments

Jared M. Diamond

SECONDARY sexual structures may serve not only as signals to attract mates and dominate rivals, but also as a way of reproductively isolating one species from another. So we hardly expect one species to borrow another's ornaments. Such a discovery by Frith and Frith<sup>1</sup>, reported in *Emu*, is all the more remarkable because the particular ornament borrowed is one of the most bizarre in the animal kingdom. The observation illuminates the much debated question of whether sexual ornaments are mere arbitrary neutral characters or are valid indicators of a suitor's quality.

The structures in question are the occipital plumes of the adult male King of Saxony bird of paradise (*Pteridophora alberti*), a thrush-sized bird found only in New Guinea cloud forest. From above each eye springs a highly modified feather several times the bird's length, resembling a fine wire bearing dozens of squares of blue plastic (see figure). When the first stuffed *Pteridophora* skin reached Europe in 1894, the then-curator of birds at the British Museum (Natural History) bristled with indignation — in his view even a fool could recognize the supposed bird as a pasted-together, man-made artefact.

The borrower is Archbold's bowerbird (*Archboldia papuensis*), a little-known species occupying the same habitat as *Pteridophora*. Like other bowerbirds, male *Archboldia* construct and decorate a bower by which females select a mate. Frith and Frith found that the central mats of six out of twenty *Archboldia* bowers examined were decorated with an average of three and a maximum of six *Pteridophora* plumes. The plumes and their position were evidently highly important, because the bower-owning *Archboldia* male moved the plumes back to the centre of the mat when Frith and Frith shifted them to the edge.

For years there has been vigorous argument over whether sexual ornaments are arbitrary signals of no intrinsic value (Fisher's runaway model<sup>2</sup>), 'good' traits

serving as direct indicators of owner quality (truth-in-advertising<sup>3</sup>), or burdensome traits demonstrating the owner's ability to survive despite such an impediment



Plumes for the pinching, here on their rightful owner the male King of Saxony bird of paradise. (Painting by William T. Cooper, reproduced with kind permission.)

(Zahavi's handicap principle<sup>4</sup>). These hypotheses are normally applied to ornaments that are part of an animal's body, but they can also be applied to the ornaments with which a male bowerbird decorates his bower. What message is conveyed to *Archboldia* females by the presence of *Pteridophora* plumes in an *Archboldia* bower?

The plumes must be difficult to come by, because male birds of paradise acquire them only at an age of four to seven years and moult only once a year, and because *Pteridophora* is an uncommon species of a notoriously impenetrable habitat where moulted feathers are hard to find. Males of other bowerbird species are known to

steal prized ornaments from each other's bowers. So a female *Archboldia* finding a bower with several *Pteridophora* plumes knows at once that she has located a dominant male who is terrific at finding or stealing rare objects and at defeating would-be thieves.

Australia's satin bowerbird similarly decorates its bowers with uncommon, often stolen, blue parrot feathers, though these are not nearly so rare as *Pteridophora* plumes<sup>5</sup>. But acquisition and retention of rare objects are not the sole truthful signals by which a male bowerbird can advertise his superiority. Frith and Frith note that another species, the tooth-billed bowerbird, decorates its bowers just with leaves that are not at all rare. Among Vogelkop gardener bowerbirds, whereas one population prefers blue objects as does the satin bowerbird, another uses acorns and snail shells which are abundant. But bowers of both populations are still massive, intricately woven edifices, decorated according to a complex design with hundreds of objects, including leaves two metres long and weighing half the weight of the male himself<sup>6</sup>. It is as if a woman were to put her suitors through a sewing contest, chess tournament, boxing match and weight-lifting contest before going to bed with the winner.

The discovery by Frith and Frith therefore adds to the evidence that male bowerbirds' ornaments signal their owner's superiority through the truth-in-advertising principle. For the same reasons implicit in *Archboldia*'s choice, *Pteridophora* plumes are also prized by males of yet another New Guinea montane species: highland men, who use the plumes to decorate their headdresses. Ironically, however, the message to a female *Pteridophora* herself is probably a different one.

*Pteridophora* plumes mounted on their rightful owner surely signal male quality by Zahavi's handicap principle, because only the most superior bird could navigate his way through New Guinea's cloud forest while sporting plumes several times his own length. □

Jared M. Diamond is in the Department of Physiology, University of California Medical School, Los Angeles, California 90024, USA.

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# Solar gases in the Earth?

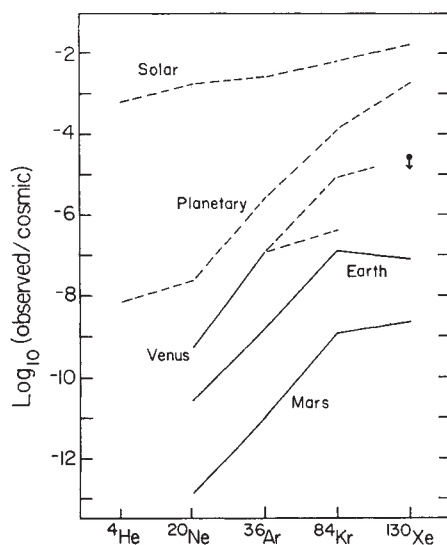
Frank A. Podosek

On page 149 of this issue<sup>1</sup>, Honda *et al.* report analyses of helium and neon in samples from the Loihi Seamount and the East Rift Zone of Kilauea on the island of Hawaii, the archetypal 'hotspot' thought to reflect an upwelling plume of deep mantle material, and a favoured sampling location for geochemists seeking to characterize the most primitive accessible materials in the Earth. Honda *et al.* conclude that their data indicate that there is a primitive 'solar' component in the Earth. More specifically, this is an isotopic identification: Honda *et al.* assert the presence of primitive terrestrial neon with the same isotopic composition as that believed to characterize the Sun. On the face of it, this may not seem particularly noteworthy, as a near identity of isotopic composition is generally believed to characterize most elements in macroscopic samples throughout the Solar System. There are exceptions to this generalization, however, notably among the noble gases. If the solar neon identification is correct, a good deal of rethinking will be in order concerning volatile inventories and atmospheric evolution for the terrestrial planets.

In the first place, neon in the Earth's atmosphere definitely does not have a solar composition: in air,  $^{20}\text{Ne}/^{22}\text{Ne} = 9.8$ , whereas in the primitive component identified by Honda *et al.*  $^{20}\text{Ne}/^{22}\text{Ne} \geq 10.9$  and presumably (see below)  $^{20}\text{Ne}/^{22}\text{Ne} \approx 13\text{--}14$ . For a variety of seemingly good reasons<sup>2</sup>, it has long been thought that the Earth's atmosphere is secondary, formed of volatiles originally incorporated in the solids that came together to form the Earth and subsequently degassed from the solid Earth as part of the process of planetary differentiation. Such a model requires isotopic identity between the atmosphere and any residual volatiles remaining in the mantle, or at least a good explanation for any difference.

The traditional approach to explaining isotopic diversity in noble-gas geochemistry is to invoke variable amounts of a nuclear component<sup>3</sup>, arising from radioactive decay or nuclear-particle interactions, added to some ancestral composition. Such effects are surprisingly common in the noble gases, primarily because their natural abundances are so low that small amounts of such a nuclear component can have a big isotopic effect (for example, in some samples, natural fission leads to pronounced changes in the isotopic composition of xenon but to negligible effects in the more abundant lanthanide elements). For neon, in particular, there are reasonably well-understood nuclear processes involving

$\alpha$ -particle and neutron interactions, attributable to uranium and thorium decay, which modify isotopic compositions in terrestrial materials. But their effect is to decrease the ratio  $^{20}\text{Ne}/^{22}\text{Ne}$  (and much more prominently to increase the relative abundance of the scarce isotope  $^{21}\text{Ne}$ ), not to increase it, so that they



Elemental abundance patterns for noble gases (atmospheric inventory divided by planetary mass) for Earth, Mars and Venus, and representative 'planetary' and 'solar' abundance patterns in extraterrestrial materials. The planetary and solar patterns are displaced to high ordinate positions for clarity; specific samples have similar elemental abundance ratios but at lower absolute abundances. (From ref. 2.)

cannot account for the difference between atmospheric neon and solar-like neon in the mantle.

Some more radical reinterpretation thus seems to be required. One possibility is to suppose that the present atmospheric neon is the isotopically fractionated residue of a more massive (but still secondary) atmosphere mostly lost by hydrodynamic processes early in the Earth's history. Such models have been considered for reasons other than neon composition, but invariably involve very large degrees of loss and make similarly extreme predictions for the elemental abundances and isotopic compositions of other atmospheric species. A second possibility also supposes hydrodynamic loss, but of a primary rather than secondary atmosphere: that is, one accreted directly as a gas rather than formed by degassing of the solid Earth. A third is to invoke heterogeneous accretion of the solid Earth and suppose the atmosphere to have formed by degassing of a late veneer unrelated to the present mantle.

Such models are possible but they are

not popular in the mainstream. Each requires the supposition of the appropriate events and boundary conditions; moreover, in models involving large changes in elemental abundances some analogies between atmospheric composition and volatile abundances in extraterrestrial materials must be dismissed as coincidence, and in models divorcing the atmosphere from the mantle a number of isotopic relationships<sup>4</sup> between atmospheric and mantle species, particularly those in relatively primitive undifferentiated mantle materials, must presumably be similarly dismissed.

Besides the question of accounting for the atmosphere if primitive terrestrial neon is solar, there is the problem of how Earth materials acquired solar neon. In extraterrestrial materials (meteorites and lunar samples) noble gases sometimes occur in the so-called solar pattern, with a characteristic elemental abundance pattern not much fractionated relative to solar composition (see figure). There are several indications that this solar abundance pattern originated from the implantation of ions in the solar wind on airless bodies such as the Moon or asteroids. Our notion of the isotopic composition of noble gases in the Sun is based primarily on analyses of extraterrestrial materials bearing the solar elemental abundance pattern. This is in reasonable agreement with direct measurements of the solar wind<sup>5</sup>. The solar pattern is not the most common, however: the characteristic occurrence of noble gases in primitive (undifferentiated) meteorites is the planetary pattern, defined by progressively greater depletion of lighter gases relative to heavier ones, by characteristic ratios spanning four to five orders of magnitude from lightest to heaviest (see figure).

In contrast to solar gases, the origin of planetary gases is a long-standing question which has never been satisfactorily resolved. The principal difficulty is not why noble gases are so depleted overall but why, under the conditions in which meteorite and terrestrial planet materials are believed to have formed, they are not depleted still more. Nevertheless, planetary patterns of noble gases are observed and it is logical to expect that they also existed in the materials of which the Earth was made. This expectation is supported by the observation that atmospheric noble gases are found in about the right amounts and proportions (see figure); this approximate match, and the strong contrast with solar proportions, is indeed the reason for the term planetary. To the extent it is possible to judge with limited data, noble gases in the atmospheres of Mars and Venus also occur in more nearly planetary than solar proportions.

There are also characteristic isotopic differences<sup>6</sup> between planetary and solar



gases, particularly in helium, neon and xenon, which are partially (but only partially) understood in terms of specific nuclear components, including those carried in presolar interstellar grains<sup>6</sup>. In planetary gas,  $^{20}\text{Ne}/^{22}\text{Ne} \approx 8.5$ . The observation that atmospheric  $^{20}\text{Ne}/^{22}\text{Ne}$  is distinct from the solar value and close to the planetary value has been taken as further evidence that the Earth's noble gases are planetary rather than solar in origin, whatever that origin may be.

If primitive terrestrial neon is actually solar, what of the other noble gases? Perhaps they should be solar as well, but not necessarily, as it is possible to superpose solar and planetary patterns (as occurs in some meteorites) so that helium and neon are primarily solar while the heavier gases are primarily planetary. If solar neon is imagined to arise by the same mechanism as in meteorites, by solar wind implantation, then cosmochemistry is faced with the problem of arranging a dispersal of pre-Earth materials long enough to acquire the necessary solar-wind exposure, and without producing the same effect in pre-meteorite materials. Or else cosmochemists are faced with the task of inventing still another mechanism for incorporating noble gases in solids.

The notion of solar neon in the mantle is not exactly new; reports of mantle neon with  $^{20}\text{Ne}/^{22}\text{Ne}$  greater than the atmospheric ratio have been made before<sup>7</sup>. The question has not received the attention it deserves, perhaps because the early results could be disputed, because of a general belief that noble gas isotopic variations could always be interpreted in terms of some obscure nuclear effect, and because some investigators simply hoped the problem would go away. As more and better data have been acquired<sup>1,8</sup>, however, the problem has grown more acute rather than subsided. The report by Honda *et al.*<sup>1</sup> does not break new ground in terms of extreme isotopic effects:  $^{20}\text{Ne}/^{22}\text{Ne}$  ratios essentially as high as the solar value (around 13) have been found<sup>9</sup> before, whereas the Hawaiian data<sup>1</sup> show only more modest enhancements above the atmospheric ratio and are considered solar only because no other Ne compon-

ent is known with  $^{20}\text{Ne}/^{22}\text{Ne}$  higher than the atmospheric value. Previously, however, the high  $^{20}\text{Ne}/^{22}\text{Ne}$  ratios have been ascribed to depleted and degassed mantle that was the source for mid-ocean-ridge basalts<sup>9</sup>. The present Ne content of this mantle is a very minor part of the total terrestrial inventory. Its neon isotopic composition could be imagined, even if with some difficulty, to be only a distorted (fractionated) representation of primordial neon. The report of Honda *et al.* is novel in that it ascribes solar neon to relatively undergassed and undepleted mantle

which, in models of atmospheric evolution, should have the same isotopic composition as air<sup>9</sup>. Their report is also novel in that they link nuclear components in  $^4\text{He}$  and  $^{21}\text{Ne}$ , both ascribed to uranium and thorium, with the primordial components in  $^3\text{He}$  and  $^{20}\text{Ne}$ , and so infer an elemental (He/Ne) ratio for primordial gas which is nicely compatible with the solar ratio. □

Frank A. Podosek is in the McDonnell Center for Space Sciences, Washington University, St Louis, Missouri 63130, USA.

## PROTEIN TRANSLOCATION

# A bacterium catches up

Tom A. Rapoport

GENERALIZATIONS in biology, where rules are the exception, are gratifying. New insight into protein translocation now offers such a satisfaction. It had seemed that the molecular mechanisms of translocation across the endoplasmic reticulum (ER) membrane of eukaryotes and the cytoplasmic membrane of bacteria were very different. But Ribes *et al.*<sup>1</sup> and Poritz *et al.*<sup>2</sup> now demonstrate similarities between the two processes, in that they have discovered a ribonucleoparticle in *Escherichia coli* that resembles the signal-recognition particle found in mammals.

In mammalian cells, most proteins translocated across or inserted into the ER membrane need the signal recognition particle (SRP), an 11S cytoplasmic particle consisting of six distinct polypeptides and a 7S RNA. The signal sequence of a nascent polypeptide is cotranslationally bound by SRP54, the subunit of SRP of relative molecular mass 54,000 ( $M_r$  54K). The complex of SRP, nascent chain and ribosome then interacts with the SRP receptor (docking protein) at the ER membrane to be followed by the actual protein transfer (see ref. 3 for review).

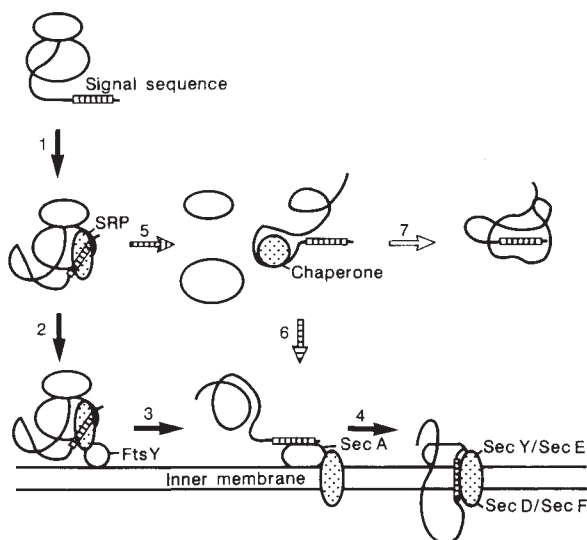
The discovery of an SRP-like particle in *E. coli* does not come as a complete surprise. Because signal sequences of bacteria and eukaryotes are interchangeable (see ref. 4), a similar mechanism of recognizing them could be expected. A 4.5S RNA and a protein of  $M_r$  48K (P48 or Ffh protein) were known to be very similar to the 7S RNA and the SRP54, respectively<sup>5,6</sup>, and to be essential for cell viability (ref. 7; G. Philips and T. Silhavy, personal communication). The 4.5S RNA was found to be on the ribosome during elongation of some polypeptide chains<sup>8</sup>. Now it has been shown that both 4.5S RNA and P48 are parts of a 10S ribonucleoprotein. The 7S RNA can bind P48 and is even able to take the place of the 4.5S RNA of *E. coli* *in vivo*, whereas the 4.5S RNA binds SRP54 and can replace

the 7S RNA in an enzymatic assay. A candidate for an SRP receptor (the FtsY protein) has also been found in *E. coli*<sup>5,6</sup>. Other eubacteria and archaeobacteria also contain at least an SRP RNA<sup>9</sup>.

To test the function in protein translocation, the number of intact SRP molecules per *E. coli* cell was lowered, or P48 was overexpressed<sup>1,2</sup>. Of the five exported proteins tested, only  $\beta$ -lactamase showed a cytosolic accumulation of its precursor well before cell death. A heat-shock response was also observed, indicative of stress probably induced by the accumulation of misfolded proteins in the cytoplasm.

Although the results do not unequivocally prove a role for the SRP-like particle in protein transport in bacteria, they provoke interesting speculations. It seems that there are several pathways for protein translocation in *E. coli* (see figure), only one of which depends on SRP. One possibility is that the SRP pathway is used by only a minor class of proteins, in contrast to the situation in eukaryotes. Still, this pathway may be the more ancient one. The SRP-independent pathways would be predominant. They involve other cytosolic factors, called molecular chaperones, such as SecB, GroEL/GroES, DnaK and perhaps trigger factor (see ref. 10 for review), which bind to precursor molecules but have no specificity for signal sequences<sup>11</sup>. These factors can partially replace one another and can bind post-translationally to completed precursor proteins, a feature distinguishing them from SRP. Among the chaperones, only SecB seems to be specific for translocation, the others having pleiotropic functions. SecB binds the precursor and transfers it to SecA, a peripheral protein of the inner membrane with an ATPase-activity, which may recognize the signal sequence<sup>12,13</sup>. SecA may be the point of convergence of the pathways. Subsequently, the process may require integral membrane

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Possible pathways of protein translocation in *Escherichia coli*. Step 1, the signal sequence has emerged from the ribosome and may bind the signal recognition particle (SRP). Step 2, the complex of nascent polypeptide chain, SRP and ribosome binds to the inner membrane, probably through FtsY. Step 3, the nascent chain is transferred to SecA. Step 4, transfer of the polypeptide through the membrane, with the involvement of the integral membrane proteins SecY, SecE and, probably, SecD and SecF. Step 5, proteins that have missed their chance of cotranslational targeting may bind a chaperone. Step 6, they may then be transferred to SecA and converge in the common pathway. Step 7, alternatively they may misfold and not become translocated. Some proteins, such as the M13 procoat, may be directly transferred across the membrane after step 5, bypassing SecA and the inner membrane proteins<sup>17</sup>. Many of the steps shown as unidirectional may in fact be equilibria.

proteins (SecY, SecE, SecD, SecF)<sup>14</sup>.

I favour an alternative possibility that would unify the function of SRP in nature. SRP-independent translocation would only serve as a salvage pathway for precursor proteins that have missed their chance of cotranslational targeting by SRP. In this case, the SRP pathway would be the basic one, alleviating constraints that particular folding characteristics of a protein might impose on its translocation. It may be assumed that all signal sequences interact with SRP to form a complex, but with different binding constants. The concentration of the complex would then determine the rate of translocation. Peptide chains with weak signal sequences (like, presumably, that of pre- $\beta$ -lactamase) may be completed in a larger proportion before SRP-dependent translocation. They may then be rescued for transport by interaction with chaperones. Alternatively, aberrant folding may make them incompetent for translocation. If the level of functional SRP is lowered, or if its membrane interaction is retarded by excess P48, proteins with signal sequences of low affinity for SRP may be affected first. Among the latter (besides  $\beta$ -lactamase) may be some which use the salvage pathway insufficiently and are essential for the viability of the cell. Death would occur before the level of SRP is lowered so far as to exert an effect on

exported proteins with stronger signal sequences. Under normal conditions, only a few proteins would use the salvage pathway and need a chaperone (SecB, for example)<sup>15</sup>. Under unfavourable conditions more proteins are forced into this pathway and heat shock proteins are called on. Adaptation of folding properties of proteins to such conditions may be common in bacteria.

According to this model, the process in eukaryotes differs only quantitatively. The eukaryotic translation mechanism is much slower and there is enough time for the growing polypeptide chain to bind SRP well before its completion. As a consequence, the folding properties of the mature portion of an exported protein will have less influence on the efficiency of translocation than in bacteria. Nevertheless, a salvage pathway may exist even in eukaryotes at least for short polypeptide chains, and a number of proteins can indeed be translocated post-translationally with the aid of chaperones<sup>16</sup>.

It is now clear that not only are the signals for protein translocation the same in all organisms, but that they are recognized by molecules (P48 or SRP54) that have been highly conserved in evolution. Given that, one may hope that other basic features of the translocation apparatus will also turn out to be universal. □

Tom A. Rapoport is in the Central Institute of Molecular Biology, Robert-Rössle-Strasse 10, 1115 Berlin-Buch, Germany.

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## Shaped charges

PLASTIC moulding is the simplest of processes. You just melt the polymer and force it into a steel mould of the shape you want. The vast expense of the mould limits you to products which can be sold in identical millions. Daedalus is now changing all this. He has been inspired by electron-beam thermoplastic recording, in which an electron beam writes a pattern of charge density onto a moving plastic tape, which then passes through a heat-softening chamber. The charged regions repel each other, deforming the tape surface into a pattern of dense ripples which can be read optically. DREADCO engineers are scaling up the process. They are passing continuously extruded plastic tube or rod through a vacuum chamber, and dumping quite ferocious patterns of charge density onto it from a battery of programmed electron guns. On subsequent heating, it expands and deforms dramatically into the shape defined by the imposed charge-pattern.

The new 'thermo-electroforming' process is a computational nightmare. Even simple symmetrical objects like bowls and buckets pose severe challenges, while DREADCO mathematicians are still struggling vainly to calculate the exact pattern of charge needed to deform a length of plastic rod into a complex object like a chair, a computer keyboard or a telephone handset. But traditional plastic-fabrication moulds are so expensive and inflexible that any way of defining a shape in software rather than steel should be worth all the pain of development.

Even better, a thermo-electroformed product can be improved or modified at any time by simple software patching, rather than making new expensive moulds. Instead of being limited to mass designs, plastics manufacturers will be able to tweak and customize their products at will. They will move up-market into specialized niches, indulging their customers with personal flourishes and elegant variations on designs which will themselves be continually optimized and upgraded.

And thermo-electroformed articles will be beautiful. Despite the specific engineering requirements, the overall shape of a plastic object is usually pretty arbitrary. Steel-mould technology has encouraged such universally tasteless styling that the very word 'plastic' has become a term of abuse. But thermo-electroforming will produce flowing, organic, integrated shapes, naturally defined and unified by the electrostatic field-equations. Good designers will cease to impose arbitrary styling on such sculptural creations. Instead they will relish the charm and economy of the shapes arising naturally from simple charge distributions. A new elegance and style will infiltrate our graceless age of plastic.

David Jones



## Mercury risk from teeth

SIR—Mills in Scientific Correspondence<sup>1</sup> estimated the amount of mercury released into the atmosphere from crematoria. But in the case of the crematorium quoted, Mills assumed that each of the 3,831 people cremated in 1989 had five amalgam fillings in their teeth, based on an estimate<sup>2</sup> that 30% of adults in the United Kingdom had lost all their natural teeth and the rest had 7.5 restorations.

When the age distribution of the population is considered, the total number of deaths in England and Wales in 1988 was 571,408 (ref. 3). Of these, 161,587 (28.3%) were under 65 years old and 409,821 (71.7%) were aged 65 and over (24% aged 65–74 years and 47.7% over 75 years). Furthermore, 57% of the population aged 65–74 years old are edentulous, whereas the corresponding figure for those over 75 years old is 80% (ref. 4). In addition, each of the 59,000 65–74-year olds who has some natural teeth has 5.7 fillings, and only 3.7 teeth have been restored in each of the 54,500 people over 75 years of age.

If we assume that all the fillings are of amalgam, then the weight of mercury released from the 65–74-year-old group is  $59,000 \times 5.7 \times 0.6$  g, or 201,780 g, and  $54,500 \times 3.7 \times 0.6$  g, or 120,990 g, from those over 75 years. The number of people under the age of 65 who died was 161,587, each of whom had approximately nine fillings. This accounts for  $161,587 \times 9 \times 0.6$  g, or 872,570 g, of mercury.

Thus, the maximum weight of mercury released annually in England and Wales is 482 kg. This is a generous estimate as not all restored teeth are of amalgam. Another fact which reduces this figure is that only 68% of the population are cremated<sup>5</sup>. Hence, the total amount of mercury released would be no more than  $482 \times 68/100 = 328$  kg. Because this mass would be released by 571,408 individuals, the average mass released from each cremation would be approximately  $482/571 \times 68/100 = 0.574$  g. Hence, for the crematorium studied by Mills, the mass of mercury released would be  $3,831 \times 482/571 \times 68/100$  g, or 2,199 g, and not 11,000 g.

Mills suggests that careful ground and air sampling programmes should be initiated to assess any possible hazard. In the case of ground contamination, one investigation<sup>6</sup> has shown that the mercury contamination in soil in close proximity to a crematorium is no greater than  $130 \mu\text{g kg}^{-1}$  (Mansfield District Council, unpublished report). It should be noted that the number of cremations in 1989 was 2,710 and that the total number of cremations since this crematorium was opened was 63,598 (ref. 7).

Although it can be assumed that no

health hazard exists to the public at large, at exposure levels below  $1 \mu\text{g mercury m}^{-3}$  in ambient air<sup>7</sup>, the threshold limit value for occupationally exposed individuals is  $50 \mu\text{g m}^{-3}$  over an 8-hour period<sup>8</sup>. In the case of crematoria, mercury may be released over an 8-hour working day, in which case the threshold limit value for this element of  $50 \mu\text{g m}^{-3}$  in ambient air might be appropriate.

M. K. BASU  
H. J. WILSON

The Dental School,  
The University,  
Birmingham B4 6NN, UK

G. KRISHNAN

Health and Safety Executive,  
2 Masshouse Circus,  
Queensway,  
Birmingham B4 7NP, UK

MILLS REPLIES—I am glad that my estimate of the amount of mercury released from crematorium chimneys is probably too large. However, in view of the importance now attached to the safe handling of even small quantities of this toxic element in laboratories and in industry, an emission of 2 kg of the vapour from low chimneys in urban areas is, in my opinion, completely anomalous and a real cause for concern. Also, this figure may be expected to rise because of the increasing popularity of cremation and improved long-term dental care of the general population until an effective non-mercurial substitute for amalgam becomes both available and numerically significant. I therefore continue to call for careful air and soil surveys around operating crematoria, with the methods and results to be published in the open scientific literature.

I also query the Basu *et al.*'s proposed threshold limit value of  $50 \mu\text{g m}^{-3}$ : because the general population (unlike a factory workforce) will include pregnant women, babies and young children, I prefer Gerstner and Huff's figure<sup>7</sup> of  $1 \mu\text{g m}^{-3}$ . A limit for mercury should certainly be incorporated in regulations governing emissions from crematoria.

ALLAN MILLS

Department of Geology,  
The University,  
Leicester LE1 7RH, UK

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## Plants without chlorophyll

SIR—dePamphilis and Palmer report<sup>1</sup> that the plastid DNA of the achlorophyllous flowering plant *Epifagus virginiana* has lost numerous photosynthetic and *ndh* genes, while retaining ribosomal RNA, transfer RNA and ribosomal protein genes, open reading frames and the inverted repeat — they ask “why does *Epifagus* maintain a plastid genome?”. We suggest that the remnant genome is necessary for biosynthesis of haem for mitochondria and other organelles, which will still be required by an achlorophyllous plant.

The available evidence indicates that the early stages of porphyrin biosynthesis in plants (leading to haem as well as chlorophyll) are restricted to plastids in non-photosynthetic as well as photosynthetic tissues. In particular, the enzymes 5-aminolaevulinic acid dehydratase and porphobilinogen deaminase (which catalyse early steps in porphyrin biosynthesis) are confined to plastids in pea leaves and *Arum* spadices<sup>2</sup>. The latter enzyme is also found in plastids of *Euglena*, streptomycin-bleached mutants of *Euglena*, and *Astasia longa*<sup>3</sup> (a morphologically similar achlorophyllous alga that, like *Epifagus*, retains a plastid genome depleted of genes for photosynthetic proteins<sup>4</sup>).

Furthermore, plant haem is labelled by glutamate (used as a precursor in the plastid) rather than glycine (used as a precursor in mitochondria from other organisms)<sup>4</sup>. Within the plastid, formation of 5-aminolaevulinic acid from glutamate for porphyrin biosynthesis requires activation of the glutamate by a plastid-encoded cognate transfer RNA<sup>5</sup>. Plastid DNA from achlorophyllous plants would therefore be expected to retain genes encoding at least this transfer RNA, RNA polymerase for its transcription and ribosomal proteins, ribosomal RNA and other transfer RNAs for synthesis of the RNA polymerase.

CHRISTOPHER J. HOWE

Department of Biochemistry,  
University of Cambridge,  
Tennis Court Road,  
Cambridge CB2 1QW, UK

ALISON G. SMITH

Department of Botany,  
University of Cambridge,  
Downing Street,  
Cambridge CB2 3EA, UK

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# Acoustic estimates of Antarctic krill

SIR—Everson *et al.*<sup>1</sup> discussed the implications of new measurements of target strength for estimating the abundance of krill in the Southern Ocean. Their conclusions were first, that previously used equations<sup>2</sup> relating target strength to physical size of these animals were greatly in error and, second, that the use of these equations has resulted in gross underestimates of krill abundance in the Southern Ocean. As krill provides the basis of a large fishery and is the main component of the diet of many marine predators, accurate estimates are essential for management of this resource. We have collected data covering a broad size range of crustacean zooplankton and micronekton, and verify and elaborate the findings of Everson *et al.* We present new target-strength-by-size relationships over the full size range of krill at the acoustical frequencies commonly used in field studies<sup>3</sup>.

We collected data at 420 kHz relating target strength to  $\log ka$ , where  $k$  is the acoustical wavenumber and  $a$  is the animal's equivalent spherical radius ( $a$  in the figure). Although both theoretical and empirical studies indicate that the relationship between these two variables is nonlinear, a linear regression model relat-

ing target strength to  $\log ka$  explains a high proportion of the observed variance ( $r^2 = 0.81$ ) in the geometric scattering region where values of  $ka$  exceed 1 (ref. 4). Some of the variance unexplained by the linear regression model can be associated with certain modal oscillations predicted by theoretical scattering models<sup>4</sup>. Until these theoretical models are better developed, the linear model is of more practical use for predicting target-strength-by-size relationships. One attractive feature of the target-strength-by- $\log ka$  relationship is that it can be manipulated, using a theoretically derived  $10 \log k$  conversion factor<sup>4</sup>, to predict how the target strength varies with animal size at a given acoustical frequency ( $b, c$  in the figure).

We note two points arising from the empirically derived relationships presented in the figure. First, the forms of the relationships imply that crustacean zooplankton and micronekton scatter sound as a function of their volume rather than their cross-sectional area. This result, although consistent with most previous studies of sound scattering by crustacean zooplankton and micronekton<sup>4</sup>, is in conflict with the equations recommended for krill by the first international BIOMASS experiment (FIBEX) acoustic working

group<sup>2</sup>. Second, predicted target-strength values for various sizes of crustacean zooplankton and micronekton are considerably lower than those predicted by the FIBEX equations. This finding verifies the general conclusions of Everson *et al.*, despite some minor differences in the results of our two studies: Everson *et al.* estimated a slightly lower mean target-strength value at 120 kHz than we do, and they included results in the 30–40-mm size range at 38 kHz.

The discrepancies at 120 kHz can be partially accounted for by differences in experimental methods and/or failure to control for orientation effects in both studies. Also, while the animals in the two studies are anatomically similar, they are of different species, making direct comparison somewhat speculative. The differences at 38 kHz are more fundamental. At this frequency, krill smaller than about 50 mm fall in the Rayleigh scattering region of the target-strength-by- $\log ka$  relationship, in which target strength decreases precipitously with decreasing animal size. This results in a reduced ability to detect smaller animals as their echo levels fall below the inherent noise of the sonar system. For this reason, we recommend that only acoustical frequencies equal to or exceeding 120 kHz be used for surveying the abundance of post-larval krill.

Thus, acoustical methods are well suited to the task of estimating krill abundance, but their use requires calibrated sonar systems and reliable target-strength-by-size relationships over the full size range of krill and at each of the acoustical frequencies being used. Although improvements can be made, the relationships presented here are the most reliable available and should enable others to interpret new acoustical survey data and to reanalyse older data sets more accurately.

CHARLES H. GREENE

Ocean Resources and Ecosystems  
Program,  
Cornell University, Ithaca,  
New York 14853, USA

TIMOTHY K. STANTON

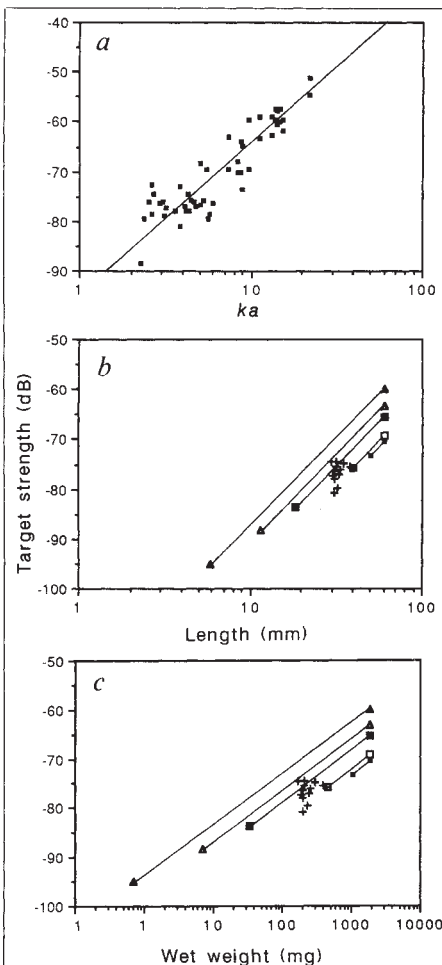
Applied Ocean Physics and Engineering  
Department,

PETER H. WIEBE

Biology Department,  
Woods Hole Oceanographic Institution,  
Woods Hole, Massachusetts 02543,  
USA

SAM MCCLATCHIE

Department of Oceanography,  
Dalhousie University, Halifax,  
Nova Scotia, Canada B3H 4J1



a. Target-strength (TS)-by- $\log ka$  relationship, with a linear regression model ( $TS = -94.93 + 30.84 \log ka$ ,  $n = 52$ ,  $r^2 = 0.81$ ) fitted to data from acoustical experiments conducted with a broad size range of living crustacean zooplankton and micronekton<sup>2</sup>. As frequency was held constant at 420 kHz during these experiments, the regression slope of 30.84 applies to variations in  $a$ , not in  $k$ . Acoustical backscattering measurements were made on individual animals using the dual-beam method for TS estimation (see ref. 4 for experimental details). b. TS-by- $\log$  length relationships predicted at five acoustical frequencies commonly used in field studies. All relationships are truncated at a minimum length corresponding to values of  $ka = 1$  and a maximum length of 60 mm. Data from ref. 1 collected at 120 kHz are included, those at 38 kHz are excluded. c. TS-by- $\log$  wet weight relationships predicted at five acoustical frequencies commonly used in field studies. All relationships are truncated at a minimum wet weight corresponding to values of  $ka = 1$  and a maximum wet weight of 1,900 mg. Data from ref. 1 collected at 120 kHz are included, those at 38 kHz are excluded. As the regression relationship reported in a is derived from data collected at a single frequency, 420 kHz, a  $10 \log k$  correction factor was applied to the relationship to predict TS values at other frequencies. This correction factor ( $10 \log k_i/k_{420}$ , where  $k_i$  is the wavenumber at the frequency of interest and  $k_{420}$  is the wavenumber at 420 kHz), was derived from a linearized version of the straight finite cylinder scattering model described in ref. 4. Values of equivalent spherical radius were converted directly to values of wet weight by the relationship:  $WW = 4.188 \times a^3$ . Values of wet weight were then converted to values of length using the published relationship:  $\log WW = -2.75 + 3.39 \log L$  (ref. 5). b, c. From left to right, lines are at 420, 200, 120, 50 and 38 kHz. Crosses, data from ref. 1 at 120 kHz.

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# Library of common protein motifs

**SIR**—One problem facing molecular biologists is the evaluation of the significance of finding a common amino-acid sequence motif in different proteins. A protein motif<sup>1,2</sup> is a list of allowed residues occurring at specific positions along a sequence and defining a protein's function or structure. These motifs, in which only a few key residues are considered, are increasingly being used to suggest relationships between proteins.

The significance of finding a motif in several proteins is often assessed from the number of motif matches expected by chance in a database search. This number can be estimated from the database frequencies of the residues in a motif (see figure for an example). But as proteins are not random strings of residues, it is not clear that this approach is reliable. Indeed, in conventional homology searches, it is well known that the statistical significance of a sequence relationship is often erroneous<sup>3-5</sup>.

Bairoch<sup>2</sup> has established a library, called PROSITE, of 337 protein motifs, based on the 15,409 proteins in the SWISSPROT14 database. Each motif in PROSITE has been defined so it occurs in nearly all members of the protein family in SWISSPROT and generally can be explained biologically—for example, the active-site sequence. In addition, PROSITE details the number of incorrect matches (false positives) when the

SWISSPROT14 database is scanned.

This information provides a benchmark for evaluating motif matches. The number of chance matches given in PROSITE can be compared with the number expected from a calculation of residue frequencies. In the figure the dominance of the boxed entries shows that the expected number of chance matches is a reliable estimate of the observed number of chance matches. This survey excluded motifs that had unknown matches.

From the figure, one obtains a probability of 0.95 (201/210) that a motif match is not a false positive when the expected number ( $E$ ) of chance matches calculated from residue frequencies is less than 0.5. Thus if one finds a known motif in a newly determined protein sequence and  $E < 0.5$ , then it is likely that this match detects a biologically meaningful relationship. Clearly, this evaluation is critically dependent on the accuracy in PROSITE of assigning true and false positives to the matches. Some of these assignments are

| Calculated expected number of random matches | Observed number of random matches as given in PROSITE |     |      |     |
|----------------------------------------------|-------------------------------------------------------|-----|------|-----|
|                                              | 0                                                     | 1-5 | 5-10 | >10 |
| <0.1                                         | 130                                                   | 1   | 0    | 0   |
| 0.1-0.5                                      | 71                                                    | 7   | 1    | 0   |
| 0.5-1.0                                      | 18                                                    | 3   | 0    | 0   |
| 1.0-5.0                                      | 20                                                    | 14  | 2    | 0   |
| 5.0-10.0                                     | 1                                                     | 8   | 2    | 1   |
| >10                                          | 0                                                     | 0   | 2    | 6   |

PROSITE motifs classified according to the chance expected number of matches and the observed number of matches. Boxed entries, agreement between expected and observed chance matches, representing 73% of the 197 motifs included. Of the 337 motifs in PROSITE, the figure excludes those restricted to a chain terminus; those that yield matches that are classified as unknown; and those that are so general that PROSITE cannot classify true and false matches. For example, one region of DNA polymerases<sup>6</sup> contains the motif (YA)-X-D-T-D-S-(LIVM). In SWISSPROT this motif was correctly identified 24 times and incorrectly found once. There are no matches when it cannot be assessed if the match is a true or false. From the frequencies of residues in SWISSPROT, the probability that one peptide has this motif is:  $(0.032+0.077) \times 0.052 \times 0.058 \times 0.052 \times 0.071 \times (0.91+0.054+0.065+0.023) = 2.83 \times 10^{-7}$ . Thus the expected number of matches in a scan of the  $4.8 \times 10^6$  residue SWISSPROT database is 1.4. Data calculated by PROMOT (ref. 7), a program to scan sequence(s) against the PROSITE motif library. PROSITE and SWISSPROT are from the EMBL data library at Heidelberg.

difficult to make but even if there are some errors, the general principle of confidence in the statistics of motif matches should remain.

Motifs must be carefully defined as in PROSITE rather than being developed *a posteriori*. It is not valid to find a weak sequence similarity between two proteins and then arbitrarily to identify common residues to establish a motif that by chance is not expected to occur in a sequence database.

MICHAEL J.E. STERNBERG

*Biomolecular Modelling Laboratory,  
Imperial Cancer Research Fund,  
PO Box 123, 44 Lincoln's Inn Fields,  
London WC2A 3PX, UK*

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## HIV and gag

**SIR**—I have recently encountered a seemingly obvious and, at first glance, simple problem: the relationship of the number of HIV particles to the amount of p24 gag gene product in culture medium or in the plasma of seropositive individuals. The measurement of p24 viral core protein by ELISA is a commonly used method to estimate the amount of HIV particles released from infected cells. But no one seems to know precisely how many virions are in 1 ml of a given culture medium containing, for example, 100 pg p24. A survey of the literature on HIV did not yield any clear answer, nor did enquiries to various companies manufacturing p24 ELISA kits. I therefore carried out a series of calculations.

The diameter of mature HIV ranges from 85 to 220 nm with an average size of 100-120 nm (ref. 1); this allows the volume of an individual sphere-shaped virion to be estimated as about  $6.97 \times 10^{-16} \text{ cm}^3$  using the formula  $4\pi r^3/3$ , where  $r$  is 55 nm. As the buoyant density of HIV particles<sup>2</sup> is 1.14-1.16 g ml<sup>-1</sup>, the mass of an individual virion is approximately  $8 \times 10^{-16} \text{ g}$ .

This, however, is the weight of an entire HIV particle, comprising the weight of core p24. As a rough estimate, the p24 gag protein, the product of one of the nine main genes encoding HIV, must represent at least one-ninth of the total viral body composition<sup>3</sup>. This estimation allocates the share of p24 to  $10^{-16} \text{ g}$ , which I call p24 constant. To find how many average-sized HIV particles are equivalent to 100 pg of p24, I divide the amount of p24 by the p24 constant. In my example, the number of

HIV particles is equal to  $100 \times 10^{-12} \text{ g}/10^{-16} \text{ g}$ , or  $10^6$ . It turns out that 100 pg p24 is equivalent to 1 million HIV particles, so 1 million infected lymphocytes in 1 ml medium have to release at least 1 HIV particle per cell to produce up to 100 pg p24 per ml culture supernatant. These numbers agree with an estimation of  $10^8$  viral particles per ml H9 culture<sup>4</sup> made by counting HIV particles in the electron microscope. H9 cells usually produce about 10 ng p24 which, using my formula, makes exactly  $10^8$  particles.

Investigations based on the physical mass of HIV and the actual number of infectious particles may be a better alternative to evaluate correctly some properties of HIV. The measurement of infectivity of virus is usually based on the arbitrary units of TCID<sub>50</sub>, which may depend on many variants such as target cell number, susceptibility, viability, doubling time, cellular cycle and so on. Because of this, TCID<sub>50</sub> tends to vary from lab to lab and from experiment to experiment. Contrary to multipartite viruses, one infectious unit of HIV should theoretically correspond to one viral particle. Thus, it may be worthwhile to correlate the infectivity of virus expressed in TCID<sub>50</sub> to the number of real viral particles.

ALDAR S. BOURINBAIR

*The Population Council,  
Center for Biomedical Research,  
1230 York Avenue,  
New York, New York 10021, USA*

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## Fish scales for human origins

SIR—Previous morphological analyses using hennigian cladistic approaches had suggested that Lake Victoria's approximately 200 species of haplochromine cichlid fishes had a polyphyletic origin<sup>1</sup>. Despite marked morphological diversity, Lake Victoria's cichlid fish flock may be monophyletic and may have arisen from one ancestral species in fewer than a million years<sup>2</sup>. Although the descendant forms have become adaptively diverse, they appear to exhibit less genetic variation in mitochondrial DNA than does the human species. I would like to suggest that the new data might help to resolve some morphological and molecular problems concerning the origin of modern humans.

Many palaeoanthropologists have held that there was insufficient time to transform the skeletal features diagnostic of more archaic European human populations into those of anatomically modern *Homo sapiens*, requiring instead an invasion of evolutionarily more advanced populations from outside the region<sup>3</sup>. But early anatomically modern *H. sapiens* populations were less divergent morphologically from late *H. erectus* or other archaic *Homo* populations than the cichlid *Ptyochromis sauvagei* is from *Astatotilapia piceatus*<sup>4</sup>.

The example of Lake Victoria also provides an opportunity to assess the timespans needed for morphological transitions. In nature, the timing of speciation events must be highly irregular and could be influenced by generation lengths, but a few simplifying assumptions might be helpful. Given an outer boundary of up to a million years for the radiation of the Lake Victoria fishes<sup>2</sup> and assuming an (unlikely) sequential, arithmetic mode of divergence, one new fish species would appear about every 5,000 years. If we use instead a geometric model of speciation, eight rounds of binary population subdivision (occurring approximately once every 125,000 years) could produce say 200 morphologically divergent populations over the same million-year timespan. Alternatively, if all the population fissioning occurred over only 200,000 years, the corresponding estimates for the intervals between splits would be 1,000 years and 25,000 years, respectively.

The longest estimate of about 125,000 years corresponds to several reliable estimates for the approximate duration of Neanderthals in the European fossil record<sup>5</sup>. The shorter estimates of 1,000 to 5,000 years are less than the limits of resolution of various dating methods used to assess the relative temporal relationships of Upper Palaeolithic hominid populations, suggesting that it would be

virtually impossible to distinguish between phyletic transformation and replacement.

Finally, what is the significance of the fact that genetic variation is lesser in fishes than among humans? If the fishes all arose within the 250,000- to 750,000-year timespan estimated for the existence of Lake Victoria yet exhibit less mitochondrial DNA variation than do humans, assumption of a constant rate of divergence of this DNA would require that the differentiation of humans in major geographical regions of the world must have occurred more than 250,000 to 750,000 years ago. Such a suggestion is consistent with a single expansion of humans out of Africa at about the *H. erectus* level, with continuity through present regional populations, rather than replacement of archaic human populations outside Africa by a second and more recent wave of anatomically modern humans.

It would be possible to escape the temporal constraints of this inference by assuming that mitochondrial DNA divergence rates are inconstant. Then, as I have observed elsewhere<sup>6</sup>, all simple bets are off and it must be concluded that mitochondrial DNA reveals as little about the timing of modern human origins as it does about the morphological transformations visible in the fossil evidence.

R. B. ECKHARDT

*Institute of Molecular Evolutionary Genetics,  
The Pennsylvania State University,  
University Park,  
Pennsylvania 16802, USA*

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## Survival test

SIR—C.H. Daugherty *et al.* (*Nature* **347**, 177–179; 1990) and R. M. May in his News and Views article on page 129 of the same issue draw attention to a case where the absence of taxonomic recognition has allowed extinction of one subspecies and reduced the likelihood that another will ultimately survive. The authors also point out that there is a need for adequate collections and for an intelligent programme of protection incorporating continuous sampling.

Until a little more than 10 years ago, the New Zealand Wildlife Department made it difficult to obtain specimens of the tuatara for most kinds of biological research; individuals were made available mainly for exhibition in zoos or for specific research projects. Sampling merely for anatomy and taxonomy was apparently not among these. I remember being

shocked when in 1979 I saw the limited amount of very old and poorly preserved material in the Wellington Museum. As Stephen's Island, less than 100 miles away, contained a population estimated at more than 100,000 specimens, it seemed desirable to have at least enough material in the museum for characterizing one population so that smaller samples might be compared to this. I am glad that the continuing efforts of many people have now permitted an improved level of sampling.

The lesson remains that protectionist impulses that limit or stop sampling do not automatically result in preservation of the species. This is certainly critical in the many parts of the world where forest, game and wildlife departments impose unrealistic bag limits on relatively common but poorly known species.

The second point is that we need to continue the maintenance of reference collections, staffed by taxonomists skilled in various techniques, now more than ever. Specialists can evaluate, add to their holdings and advise agencies regarding the most economic investment of limited money. There is a lesson here for the fate of the Natural History Museum in London, because it is said that its effort is to be diverted to projects that do not require reference collections and do not ultimately contribute to the maintenance and protection of our shrinking global diversity.

CARL GANS

*Department of Biology,  
The University of Michigan,  
Ann Arbor,  
Michigan 48109, USA*

## Earlier light

SIR—In their report on the use of azobenzene derivatives as the basis of photochemical information-storage devices<sup>1</sup>, Liu *et al.* made no reference to earlier work by myself and N. Doddapaneni<sup>2</sup>. In that work, we also studied the electrochemical behaviour of *cis* and *trans* azobenzenes, their interconversion under light irradiation and their electrochemical reduction to hydroazobenzenes.

This study, together with our related work on polycyclic aromatic quinones<sup>3</sup> was undertaken with the explicit objective of developing organic systems capable of storing and later releasing light energy.

G. KLOPMAN

*Department of Chemistry,  
Case Western Reserve University,  
Cleveland,  
Ohio 44106, USA*

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# Europe flying high

Desmond King-Hele

**Europe in Space.** By Guy Collins. Macmillan: 1990. Pp.235. £35.

EUROPE was slow to launch itself into space, lagging behind the two superpowers, as they used to be called. Now, deep in debt or economic turmoil, are they perhaps declining into superweaknesses? If so, can it be that tortoise-Europe will come to the forefront? These wider questions are immediately provoked by the optimistic tone of Guy Collins' book, though he himself does not address such issues. His history of Europe in space runs through the fumbleings of the early 1960s, chronicles the achievements of the 1970s and 1980s, and surveys the projects due to become reality in the 1990s. Despite a few culpable omissions, the book can be welcomed as a plain and level-headed account of the subject, and the optimistic bias is quite justified in view of the past and probable future European exploits in space.

The early history of Europe in space falls quite helpfully into decades. In 1960 Britain was in the premier position, possessing a liquid-fuel ballistic missile, Blue Streak, about to be discarded as a weapon but capable of launching a one-ton satellite into a low orbit, if fitted with suitable upper-stage rockets, such as the smaller UK Black Knight rocket. This was the moment when other European countries began taking an interest in space-launching projects, and the European Launcher Development Organization (ELDO) was set up in 1962 with the aim of building a satellite launcher called Europa, with Blue Streak as first stage, a French second stage and a German third stage. There were successful flights with Blue Streak and dummy upper stages in 1964 and 1965, but all attempts with the complete launcher failed because of defects in one or other of the upper stages. Blue Streak always performed well, and always in vain: the British were somewhat annoyed. A new design, Europa II, also failed on its first flight in 1971, and this can be seen as the virtual death-knell for ELDO. The future of Europe in space might have been different if just one of the flights had succeeded; as it was, ELDO was dogged by bad luck and consequently remains a historical relic of the 1960s.

Meanwhile an independent French national programme had begun in 1961, leading to the first French national launch by a Diamant rocket in 1965. An inde-

pendent British launcher, Black Arrow, was also developed later in the 1960s, and in 1971 it successfully launched the Prospero satellite. Much smaller than Blue Streak, Black Arrow was a low-cost project based on the earlier Black Knight rocket: despite the commercial possibilities, however, Black Arrow was cancelled

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Blue Streak — always performed well, just before the Prospero launch. The success of France and Britain with national launches contrasted with the repeated failures of Europa.

The European Space Research Organization (ESRO) was set up in 1962 just after ELDO, with the aim of designing and constructing scientific spacecraft for Europa to launch. ESRO was a great success, but the launching of its spacecraft had to be done by US rockets, beginning in 1968 and continuing through the 1970s.

In the early 1970s the French proposed a new launcher, eventually called Ariane: at that time West Germany was more interested in constructing Spacelab, a manned laboratory to be launched by the US Space Shuttle. In 1973 the European Space Agency (ESA) was founded, taking charge of these two projects, taking over ESRO and some remnants of ELDO, and also promoting a range of spacecraft for science and 'applications' (weather and communications satellites, for example).

The first launch of Ariane was on 24 December 1979, and the launches continued through the 1980s into the 1990s. The proportion of failures has been quite high (four of the first 18 failed), but the effects were less damaging because the failures were spread in time. So Ariane is now seen as a most successful launcher, with a full order-book.

The European Space Agency is in many respects an admirable organization, and its first Director-General, Roy Gibson, deserves some of the credit. ESA operates openly, canvasses the opinions of a wide range of scientists and is free of military influence; it adopts fairly rational programmes, arrived at by fairly democratic procedures, and it perseveres with these programmes, rarely imposing arbitrary changes. It can be criticized for being inefficient, fussy, over-bureaucratic, expensive and sometimes over-ambitious, but these faults do not seriously tarnish its shiny image. The present plans of ESA, quoted at length in an appendix to the book, were decided at a ministerial conference at the Hague in November 1987, when Britain's intransigent condemnation of the programme had the miraculous effect of uniting all the other countries in support of it.

Two costly features of the current ESA programme are cooperation with the United States in providing an element of NASA's international space station, and the development of the Hermes manned spacecraft to be launched by a new Ariane-5 rocket. Both these projects are of high risk. The space station may never materialize if the US debt problems escalate; and the rationale of Hermes is shaky — the Europeans cruising round the Earth may hope to bask in glory, but will they do anything useful? In reply it is only fair to say that ESA has triumphed before with high-risk projects, such as the Giotto probe to Halley's comet. Also the perception of failure is subjective not rational, as is shown by Spacelab: its huge cost makes it a candidate for the title of the biggest white elephant ever, because it only flew three times and many scientists thought the experiments on those flights rather trivial. Guy Collins, on the other hand, takes a more positive line, saying that Spacelab was valuable in giving European astronauts a taste of life in orbit, and in inspiring the ambitious plans for the 1990s. The latter, it should be said, also include an extensive range of scientific spacecraft under the programme Horizon 2000, and many applications satellites, in addition to the high-risk projects already mentioned.

The book covers all these events and ideas in reasonable detail and describes the individual spacecraft constructed by

ESRO and ESA. The treatment is not always balanced: for example, there is much about the French national programme and the satellites launched, and nothing about the (smaller) British programme, with no mention of Black Arrow, Prospero or the six Ariel satellites. There are some errors too, but the book can be recommended as generally reliable. □

Desmond King-Hele, 3 Tor Road, Farnham, Surrey GU9 7BX, UK.

## Bee warned

Thomas Seeley

**Anatomy of a Controversy: The Question of a "Language" Among Bees.** By Adrian M. Wenner and Patrick H. Wells. *Columbia University Press: 1990. Pp. 339. \$63.50.*

IN the 1960s a major controversy arose in the field of animal behaviour. At issue was the mechanism by which a honeybee recruits nestmates to a rich source of food. Since the 1940s it had been understood, from the work of Karl von Frisch, that one bee recruits others to rewarding flowers by means of both dances that she performs — which contain information about the direction and distance of the flowers — and floral odours that she bears — which further specify the flowers. Adrian Wenner and his colleagues challenged this view, arguing instead that recruitment occurs solely by odours, with recruits learning to recognize the odours borne by a dancing bee and then searching for the source of these odours. This challenge to the traditional view of bee recruitment, with its implication that bees cannot communicate direction and distance information, triggered intense debate, much of it laced with hostility toward Wenner. Why did this happen? What was the outcome? How should we now view the dances of bees? These are the main questions addressed in this book.

Two individuals from one side of a controversy cannot be expected to provide a reasoned, detached analysis of the controversy, and we certainly do not get such an analysis in this book. Indeed, it contains such a distorted account of the controversy that its primary value is to provide a window on the minds of Wenner and Wells, through which some future historian of science can peer to prepare an accurate account of this debate. The most serious distortion comes in the presentation of the dance-language hypothesis which, according to Wenner and Wells (see pages 63–64), ascribes no importance whatsoever to odours in recruitment. This is crucial to their position because they then argue that if they can demonstrate

that odours play a role in recruitment (which, of course, they can), then they have falsified the dance-language hypothesis. But their version of the dance-language hypothesis is a straw man. In both his 1950 and 1967 books, von Frisch explicitly discusses the importance of odours in enabling recruits to locate the recruitment target once they reach its general vicinity. Hence virtually all the evidence that Wenner and Wells muster to refute the dance-language hypothesis is actually consistent with von Frisch's view of the dance language.

Many other serious errors of scholarship further erode the credibility of this book's analysis of the dance-language controversy. One example that illustrates the depth of these errors occurs on pages 98–99, where the authors greatly misrepresent the classic 1944 experiment of von Frisch which first suggested that bees can communicate distance information. First, they claim that von Frisch did not report the numbers of bees arriving at the feeding station for all five trials, even though he does do so, in the footnotes. More importantly, they claim that the results of this experiment indicate that "all was not well" for the dance-language hypothesis because the recruit counts were lower at the feeding station (the recruitment target) than at the test station (a scented plate 30–100 metres from the feeding station). A careful reader of von Frisch's own account of these experiments will realize that there is no problem here. The recruit counts at the feeding station represent the number of captures of bees there, but the recruit counts at the test station represent the number of approaches by bees to the test station, which certainly exceeds the number of bees visiting the test station because a single bee can make many approaches. Comparing the two sets of numbers is meaningless, something which von Frisch recognized (hence he reported the feeding station counts only in footnotes), but which Wenner and Wells failed to discern.

Specialists in insect behaviour will also be astonished by numerous surprising claims: such as that the dance-language hypothesis is anthropomorphic (page 63), does not mesh well with mechanistic explanations of behaviour (page 74), and was negated by Maurice Maeterlinck's observations (page 53); that in his 1950 book von Frisch failed to distinguish between newcomers and experienced bees at a feeder (page 84); that the acoustic signal in dances is an anomaly for the dance-language hypothesis (page 118); and that Wenner and his colleagues were the first to discover that bees learn to associate odours in the hive with food at a feeder (page 119). Also, specialists are not likely to be convinced by the evidence presented in support of the odour-search hypothesis, virtually all of which (recruit-

ment dependence on odour, inefficiency of recruitment, long recruitment times, and so on) is consistent with the hypothesis that bees use dance information to find the general vicinity of a recruitment target and then use odour information to pinpoint the target.

Today, some 20 years after the start of this controversy, few students of animal behaviour doubt that bees share information about the location of rich food sources by means of their dances. Wenner and Wells attempt to show that this continued acceptance of the dance-language hypothesis reflects sociopolitical forces maintaining a traditional hypothesis, and that the empirical support for this hypothesis is weak. Sociological factors play a role in every scientific debate, and this book sheds light on those involved in this controversy. But this book's presentation of the biological issues and experimental evidence pertaining to the dance-language controversy is inaccurate and misleading. Readers beware. □

Thomas D. Seeley is in the Section of Neurobiology and Behaviour, Cornell University, Ithaca, New York 14853, USA.

## Rocky treasure

Jake Hancock

**Solnhofen: a Study in Mesozoic Palaeontology.** By K. W. Barthel, N. H. M. Swinburne and S. Conway Morris. *Cambridge University Press: 1990. Pp. 236. £36, \$59.50.*

THE Solnhofen Limestone was the first example of a sediment that was recognized to contain exceptionally well preserved fossils and hence an extraordinary range of organisms which are normally not preserved as fossils at all — a *lagerstätte* in current jargon. Thanks to *Archaeopteryx* — still the earliest known bird — the fame of this limestone has spread beyond scientists to educated people world-wide. Anybody outside Germany wanting to find out more about the petrology of the limestone and its general range of fossils would have been in difficulties. Moreover, Barthel's original book pre-dated the vital research by Keupp on the microfacies. Geologists are now indebted to Nicola Swinburne and Simon Conway Morris for an excellent general survey in English, which incorporates the considerable body of research by German sedimentologists and taphonomists during the last 25 years.

Roughly half the book is a systematic survey of the fossils known from the limestone, which includes good photographs of all the major taxa. But one is discouraged from getting much further into the taxonomy. Many of the famous publications on Solnhofen fossils, mentioned in



the introductory chapter, are not listed in the bibliography. There is the anomaly that captions to illustrations give full specific names but are listed by genus alone in the faunal list of the appendix.

For the general geologist the greater interest will be in the 40 per cent of the book on the petrology, facies distribution, palaeoecology and taphonomy. Most people know the rock only from the fossils in museums. In his classic study of the London specimen of *Archaeopteryx*, de Beer referred to the sediment as a 'lithographic slate'. In fact only a small percentage is usable for lithography; the famous fossils are even rarer in quarries that yield good lithographic stone and do not occur inside slabs that can be used for lithography. Although some of the rock has been used as a roofing-slate, it is obviously far from being a slate in the geological sense. The authors use the descriptive German name '*plattenkalk*' (slabby limestone), but rightly emphasize that there are two principal facies vertically, flinz (a pure micritic limestone) and faule (argillaceous micritic limestone), and that there are important variations from area to area, with some ten local basins of *plattenkalk* amongst the Tithonian reefal deposits. There is still disagreement on how much of the micrite is coccolithic material. The discussion on the wonderful preservation of fossils is so detailed that they can come to no totally satisfactory explanation. Barthel's own model, involving turbidity currents, which certainly does not fit all the basins, is shown to be untenable because many of the fossils themselves have orientations incompatible with bottom currents. One thing is certain: the bottom waters were lethal to most organisms. Only animals exceptionally resistant to adverse salinity or a shortage of oxygen, like the bivalve *Solenya* or the xiphosuran *Mesolimulus*, could survive for perhaps a few hours.

The authors favour a high salinity environment, which could both kill and preserve, but they cannot quote an exact modern analogue. Indeed, it may be questioned if the water could have been so lethal and so good at preservation with a salinity below that at which gypsum precipitates. They are somewhat sceptical of Keupp's hypothesis of preservation by cyanobacterial smothering, in part because a comparable *plattenkalk* facies in the Upper Cretaceous of Lebanon, which preserved the only known Mesozoic octopod, was deposited at too great a depth for cyanobacteria. The authors' brief discussion of other *plattenkalks* in their last chapter shows that they represent a variety of environments and hence probably a variety of means of preservation — it could have been cyanobacteria for Solnhofen. The arguments continue. □

Jake Hancock is in the Department of Geology, Imperial College, London SW7 2BP, UK.

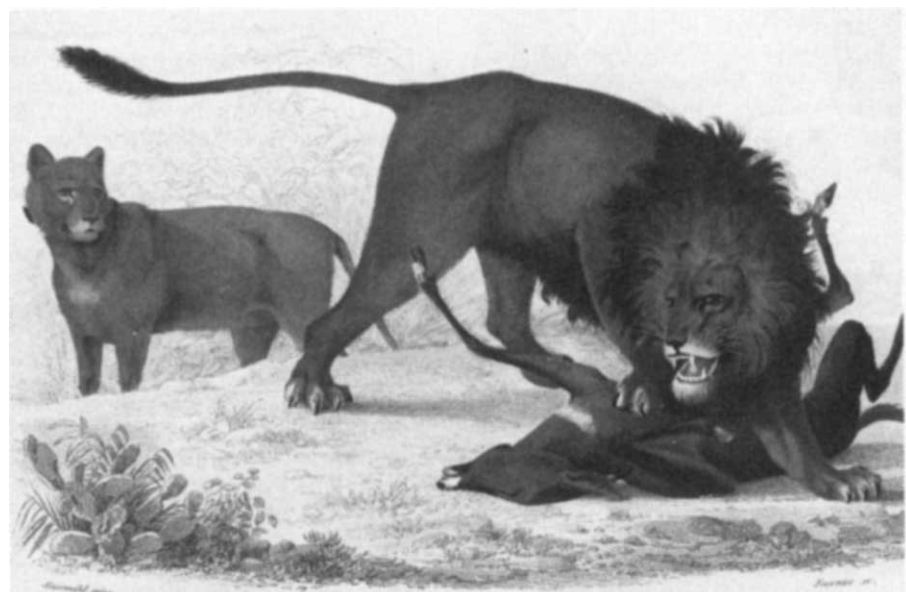
## Animated nature

Colin Patterson

**History of the Natural World.** By Oliver Goldsmith. *Studio Editions, London: 1990. Pp. 256. £17.95.*

LIKE his friend Samuel Johnson, Oliver Goldsmith was a literary drudge: today's tenants of his world of publish or perish well understand the profession. Goldsmith is best known for a poem, *The Deserted Village*, a play, *She Stoops to Conquer*, and a novel, *The Vicar of*

florid binding looks like the 1850s. I see that I paid 7 shillings and 6 pence (the equivalent of 37 pence, or 75 cents in today's money) for the set, in the happy days before secondhand booksellers called themselves antiquarians and became entrepreneurs. I suppose I bought the books because they were cheap and because both text and plates were nice, if quaint. The same excuses will do well enough for this new edition. It is a large-format abridgement, illustrated by about 125 colour plates, many of them full page, and hundreds of black-and-white vignettes. The colour plates are mostly from Charles d'Orbigny's *Dictionnaire*



The Lion — "His figure is striking, his gait proud, and his voice terrible."

Wakefield. The rest of his substantial output is hackwork of one sort or another, including *An History of the Earth and Animated Nature*. Goldsmith wrote *Animated Nature* during the last five years of his short life (1728–74), having contracted to write an eight-volume work on natural history in 1769, for 100 guineas a volume. In those years, he also wrote a history of England (for £500), a history of Greece (for £250) and *She Stoops to Conquer*, which made him at least £500. These were substantial sums at a time when Goldsmith was buying a year's board and lodging for £50; nevertheless, his best friend Joshua Reynolds reported that Goldsmith died owing at least £2,000. If he was a hack, he was a successful and profligate one.

The eight volumes of *Animated Nature* were first published three months after Goldsmith's death. The book turned out to be the eighteenth- and nineteenth-century equivalent of today's best-selling money-spinners in natural history. It went through at least 25 editions, the last of them in 1876, more than 100 years after the first. My own six-volume edition, which was lain half-forgotten for years in a lavatory cupboard, is undated but the

*Universel d'Histoire Naturelle* (1839–49), and are good reproductions of high quality originals. The vignettes are from various sources, including Thomas Bewick and Goldsmith's first edition. There is no real problem with importing illustrations like this, because Goldsmith wrote prose and every publisher of the work has added pictures more or less at random.

Then what of the text? Goldsmith was no learned naturalist, but a compiler, and his main source for the shape and substance of his work was Buffon's *Histoire Naturelle*, published in Paris from 1749 onwards. By Goldsmith's time, 16 volumes of Buffon had appeared, covering the Earth, humanity, mammals and a bit about birds. Goldsmith plagiarized Buffon shamelessly as far as the ostrich and dodo, but beyond that he was on his own. He devoted about a quarter of his space to the history of the Earth and to humanity, half to mammals and birds, and the rest to fishes (which include whales and such 'shellfishes' as lobsters, tortoises and molluscs), reptiles, insects (including spiders and leeches), and zoophytes (worms, echinoderms, coelenterates). Obviously, Goldsmith was no systematist, and he wrote of John Ray's system of

Taken from History of the Natural World.

mammals "We see, from this sketch of division and subdivision, how a subject, extremely delightful and amusing in itself, may be darkened and rendered disgusting." That passage is missing in this new abridgement, as is the whole of Goldsmith's text on the Earth and humanity. One can see why his account of human races is left out, for it is pretty strong stuff for today's taste (some of it evidently seemed strong to Goldsmith, since he dropped into Latin for the racier bits). As for the remainder — mammals, birds, testaceous fishes and so on — it is all neatly abridged here; authors who have got beyond rejection by *Nature* will know that even the best hackwork can always stand substantial cuts. I cannot disagree with Goldsmith's hope that his work "may be found an innocent amusement for those who have nothing else to employ them, or who require relaxation from labour." □

Colin Patterson is in the Natural History Museum, London SW7 5BD, UK.

## Safety control

Daniel S. Greenberg

**The Fifth Branch: Science Advisers as Policymakers.** By Sheila Jasanoff. Harvard University Press: 1990. Pp. 302. \$27.95.

CHEMO-REGULATORY drama is America's contribution to political stagecraft.

The substances at issue may be as different as pesticides and food colourings. But the elements of plot are similar: New or rediscovered research suggests a menace in public exposure to what, ironically, was intended to be a beneficence. An alarm is sounded by a public guardian, official or self-appointed. And suddenly, a previously obscure product soars to prominence on allegations of risk to health. The manufacturer indignantly protests that a human would have to ingest brooding-nagian quantities to suffer the misfortunes of sacrificial rodent consumers — a contention scornfully disputed by public-interest organizations. A film celebrity earnestly denounces the product. Meanwhile, the regulatory system is set rolling. The federal agency responsible for regulating the product summons scientific specialists to assess the risk. Charges of bias and conflict of interest are raised. Litigation ensues, suspicious congressmen call hearings, and nimble journalists hurry in quest of wrongdoing.

Why is it not possible for science to sort these things out with the precision and finality attributed to it by impatient politicians, anxious citizens — and self-confident scientists?

That is the central topic of this provoca-

tive and original work by a leading scholar of the role of scientists in regulatory affairs, Sheila Jasanoff, Director of the Cornell University Program on Science, Technology and Society. The 'fifth branch' in her formulation consists of the hundreds of advisory committees that serve US government regulatory agencies; the latter are the fourth branch, which modern needs have appended to the original Constitutional trio of executive, legislature and judiciary.

The author's principal analytical tools are, first, the sociological concept of "the social construction of science", which holds that scientific 'truth' is a product of society rather than a reflection of "what is out there in nature"; and, second, 'boundary' setting, the process whereby matters in dispute are delineated as the responsibility of scientists or policymakers.

Focusing on the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA), Professor Jasanoff is a confident guide to some of the most tortuous regulatory conflicts of recent years — eponymously known by their chemical designations, marketplace names, or statutory titles: Alar, formaldehyde, aspartame, ethylene dibromide, dicofol, propranolol, 2,4,5,-T and the Clean Air Act in relation to ozone and carbon monoxide.

Reflecting a statutory requirement, or a faith or hope that science can be sifted from politics in regulatory strife, the agencies in each instance solicited advice from panels of experts. But, as Professor Jasanoff points out, "Evidence of consultation with expert committees rarely proved sufficient to silence controversy", the reason being that "the questions regulators need to ask of science cannot in many instances be adequately answered by science." In the case of the plant-growth regulator Alar, conflicting scientific assessments led to what the author refers to "as a complete breakdown of scientific authority". The final resolution, discontinuation by the manufacturer, was prompted by a surge of public alarm arising from a television report describing Alar "as the most potent cancer-causing agent in our food supply".

*The Fifth Branch* stresses that, although regulators hopefully seek scientific guidance, the problems they present to their advisers are not the common grist of scientific problem-solving: How much risk is too much? What trade-offs are permissible between public safety and economic loss? How should regulation proceed in the presence of ambiguous or conflicting scientific and medical data? And what is to be done when experts disagree?

Attempts by regulators to extract usable answers from the orthodox processes of science almost invariably lead to contention, Professor Jasanoff argues, because these questions necessarily in-

volve social and political values and personal judgements. "Regulatory science", she observes, in contrast to conventional laboratory research, "is more often done at the margins of existing knowledge, where science and policy are difficult to distinguish and claims are backed by few, if any allies, or black boxes" — that is, undisputed facts or claims.

Peer review, the standard tool of the sciences for awarding funds and screening manuscripts, is commonly looked to by the "technocratic reformers of regulation" as a way out of regulatory morasses. But the author reminds us that science itself is discovering, through scholarly scrutiny, that peer review "is not the objective, dispassionate process that its advocates represent it to be."

Despite the battering that scientific advice has often suffered on regulatory battlefields, Professor Jasanoff says the advisory process has gained in political sophistication and is becoming more assured and effective in assisting its federal patrons. "The realities of scientific advice at EPA and FDA", she concludes, "contradict many of the myths and preconceptions that have grown up around this relatively unstudied process. The notion that scientific advisers can or do limit themselves to addressing purely scientific issues, in particular, seems fundamentally misconceived. Other common myths — for example, that scientists are always conservative in assessing risks or that advice is merely a pretext for delaying decisions — also seem exaggerated. Rather, the advisory process seems increasingly important as a locus for negotiating scientific differences that carry political weight . . . The primary concern for regulators, then, is not how to guard against capture by science but how to harness the collective expertise of the scientific community so as to advance the public interest."

The author's observation post is far above the regulatory fields of fire, and her account conveys almost nothing of the squalid dealings and political connivings common to regulatory warfare. In that respect, it has a remote, antiseptic quality, as though relating events of another era or civilization. Professor Jasanoff seems to have sensed this, for in a passing reference to 'political manipulation' of advisory committee appointments, she states: "Here, corrective action by Congress may be helpful, though the issue requires fuller investigation than it has received in this book."

Professor Jasanoff has pioneered the exploring of the workings of the gears and sprockets of the Fifth Branch. Let us hope she now turns her skills and understanding to its politics, particularly the under-  
side. □

Daniel S. Greenberg, 3736 Kanawha St. N.W., Washington, D.C. 20015, USA.



# The GTPase superfamily: conserved structure and molecular mechanism

Henry R. Bourne, David A. Sanders & Frank McCormick

GTPases are conserved molecular switches, built according to a common structural design. Rapidly accruing knowledge of individual GTPases—crystal structures, biochemical properties, or results of molecular genetic experiments—support and generate hypotheses relating structure to function in other members of the diverse family of GTPases.

A COMMON structural design and shared molecular mechanism distinguish proteins in the GTPase superfamily. Each protein in the family is a precisely engineered molecular switch that can change its affinities for other macromolecules. Turned on by binding GTP and off by hydrolysing GTP to GDP, the switch mechanism is remarkably versatile, enabling different GTPases to sort and amplify transmembrane signals, direct the synthesis and translocation of proteins, guide vesicular traffic through the cytoplasm, and control proliferation and differentiation of animal cells. As targets of mutation and microbial toxins, GTPases have pivotal roles in the pathogenesis of cancer and infectious diseases.

Increasingly rapid advances during the past five years have begun to lay bare the three-dimensional structure of the GTPase switch and to show how the switch mechanism works. Crystal structures of two GTPases, EF-Tu and p21<sup>ras</sup>, have been determined<sup>1–7</sup>. In combination with biochemical and biophysical approaches, molecular genetic experiments have assigned discrete functions of several GTPases to specific structural elements and even to individual amino acids.

The striking conservation of structure and mechanism among different GTPases has prompted this review. We shall focus on three different classes of GTPases: (1) the 21K (relative molecular mass, 21,000) products of the *ras* oncogenes and proto-oncogenes (p21<sup>ras</sup>); (2) GTPases used in ribosomal protein synthesis, exemplified by bacterial elongation factor (EF) Tu; and (3) the  $\alpha$  subunits of the heterotrimeric signal-transducing G proteins. The comparative approach will serve not only to highlight aspects of structure and mechanism widely shared among the GTPases, but also to generate experimentally testable hypotheses.

We begin with a brief account of the basic GTPase cycle, as a framework for linking biochemical function to structure. Then we describe the best resolved three-dimensional structure of a GTPase, that of p21<sup>ras</sup>. Guanine nucleotide binding domains of other GTPases resemble that of p21<sup>ras</sup> in amino-acid sequence and also—almost certainly—in three-dimensional architecture. Finally, we compare molecular mechanisms responsible for each step of the GTPase cycle. Whether we consider exchange of GTP for GDP, GTP-induced changes in protein conformation, or GTP hydrolysis, the underlying theme is the same: in each case a fascinating variety of knobs and whistles regulates the GTPase machine, while the core mechanism persists unchanged.

## The GTPase cycle

The GTPase cycle (Fig. 1) includes three conformational states of the protein. Release of bound GDP converts the 'inactive' state of the protein into a quite transient 'empty' state. Under ambient conditions in the cytoplasm, GTP is more likely than GDP to enter the empty guanine nucleotide binding site and upon binding GTP the protein takes on an 'active' conformation, which reverts to the GDP-bound inactive state when GTP is hydrolysed. In many (but not all) GTPases, the intrinsic rate constants of GDP release ( $k_{\text{diss-GDP}}$ ) and GTP hydrolysis ( $k_{\text{cat-GTP}}$ ) are quite low ( $<0.03 \text{ min}^{-1}$ ). These are increased by

either of two classes of regulatory proteins: the guanine nucleotide release proteins (GNRPs), which catalyse release of bound GDP, promoting its replacement by GTP, and the GTPase-activating proteins (GAPs), which speed up GTP hydrolysis, the irreversible step in the cycle. In principle, either a GNP or a GAP can regulate cellular function of a GTPase by controlling the relative concentrations of its active (GTP-bound) and inactive (GDP-bound) forms. For a fuller account of the GTPase cycle and its diverse cellular functions in different GTPases, see ref. 8.

## 3D structure and sequence motifs

Crystal structures of p21<sup>ras</sup> (refs 3–5, 7) and the guanine nucleotide binding domain of bacterial EF-Tu<sup>1,2</sup> are remarkably similar. The overall  $\alpha/\beta$  topologies of the two domains are the same, and their polypeptide backbones appear for the most part superimposable<sup>9</sup>, despite the fact that their primary structures are only 30% identical.

Figure 2a depicts the better resolved GTPase structure, that of p21<sup>ras</sup>, in its GTP-bound form. The protein's hydrophobic core comprises six strands of  $\beta$  sheet, which are connected by hydrophilic loops and  $\alpha$  helices. Five regions of the polypeptide chain, all associated with loops on one side of the protein, merit special attention. These regions, highlighted in Fig. 2a and designated G-1 to G-5, are critical in GDP/GTP exchange, GTP-induced conformational change, and GTP hydrolysis. Figure 2b schematically depicts bonds between bound GTP and specific amino acids in these five regions. Table 1 shows amino-acid sequences of regions G-1 to G-4 in representative GTPases from different families.

**G-1 region.** The amino-acid sequence motif, GX<sub>4</sub>GK(S/T), (single-letter code), of this region (residues 10–17 in p21<sup>ras</sup>) appears with variations in many nucleoside triphosphate-

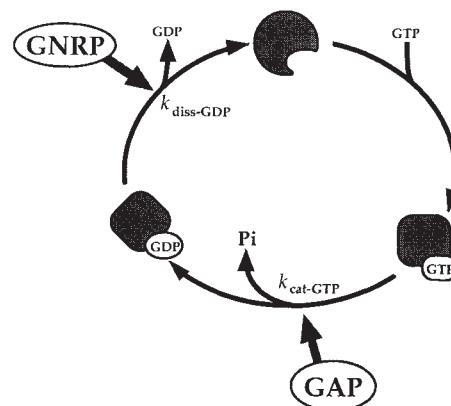


FIG. 1 The GTPase cycle, in which  $k_{\text{cat-GTP}}$  and  $k_{\text{diss-GDP}}$  are regulated, respectively, by GTPase activating proteins (GAPs) and guanine nucleotide release proteins (GNRPs).

utilizing enzymes. X-ray structures of p21<sup>ras</sup> show this region as a loop in which main-chain amide hydrogens of several amino acids, plus the  $\epsilon$ -amino group of Lys 16, form bonds with the  $\alpha$ - and  $\beta$ -phosphates of GTP or GDP<sup>4,5,7</sup>. The loop connects a hydrophobic  $\beta$  strand,  $\beta$ 1, with an amphipathic  $\alpha$  helix,  $\alpha$ 1 (Fig. 2a). The three-dimensional shapes of this region are identical in the GDP- and GTP-bound forms of p21<sup>ras</sup>.

**G-2 region.** This region (residues 32–40 in p21<sup>ras</sup>) includes the N terminus of the second  $\beta$  strand and the loop that precedes it. GTP binding changes the conformation of this loop, in part by changing the orientation of a critical threonine residue, located at position 35 (see below). Crystals of p21<sup>ras</sup> bound to a GTP analogue, Gpp(NH)p, contain<sup>7</sup> a  $Mg^{2+}$  ion that is coordinated to oxygens of the  $\beta$ - and  $\gamma$ -phosphates of GTP and to the side-chain hydroxyls of Thr 35, as well as Ser 17 from region G-1. This  $Mg^{2+}$  is essential for GTP hydrolysis. Amino-acid sequences corresponding to the G-2 region are highly conserved within each GTPase family, but not between different families (Table 1). Each putative G-2 sequence contains a conserved threonine residue, which is highlighted in Table 1.

**G-3 region.** The DX<sub>2</sub>G sequence motif of this region (residues 53–62 of p21<sup>ras</sup>) is conserved in all GTPases (Table 1). The invariant aspartate (position 57 in p21<sup>ras</sup>) binds the catalytic  $Mg^{2+}$  through an intervening water molecule<sup>7</sup>, while the amide proton of the invariant glycine (position 60) forms a hydrogen bond with the  $\gamma$ -phosphate of GTP. Conformations of amino acids 60–63 and the downstream  $\alpha$  helix ( $\alpha$ 2) differ dramatically in GTP- and GDP-bound forms of the protein (see below).

**G-4 region.** The characteristic sequence motif of this region (residues 112–119 of p21<sup>ras</sup>) consists of four hydrophobic or apolar amino acids followed by (N/T)(K/Q)XD; in p21<sup>ras</sup> this

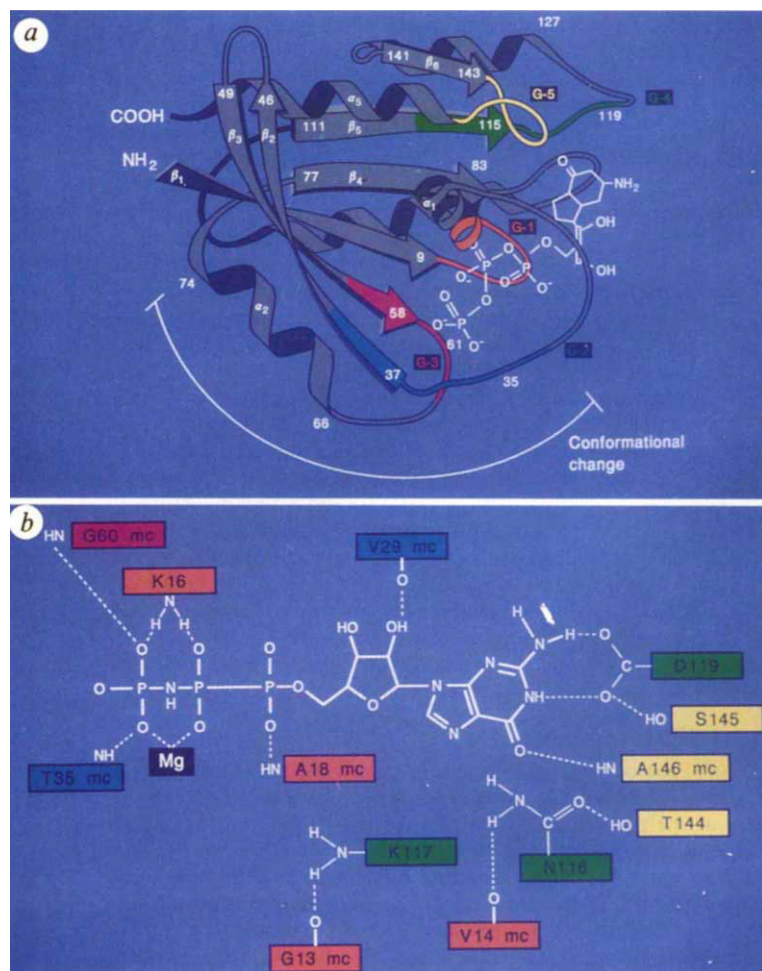
corresponds to a hydrophobic  $\beta$  strand preceding a hydrophilic loop. Carboxy oxygens of Asp 119 form hydrogen bonds with groups on the guanine ring, and amide protons of Asn 116 and Lys 117 stabilize the guanine nucleotide binding site through hydrogen bonds to residues 13 and 14 in the G-1 region (Fig. 2b). **G-5 region.** Residues 144–146 of p21<sup>ras</sup>, in a loop between the sixth  $\beta$  strand and the  $\alpha$ 5 helix, interact with the guanine nucleotide (for the most part) indirectly, through hydrogen bonds that stabilize side chains of Asn 116 and Asp 119 in region G-4 (Fig. 2b). Perhaps because this region has only one direct contact with GTP (through the main chain amide of Ala 146), it cannot be unambiguously identified by amino-acid sequence in all the subfamilies of GTPases. The G-5 region is probably conserved in all the small (20–35 K) eukaryotic GTPases, as indicated by a conserved sequence motif—OOE[A/C/S/T]SA(K/L)—in which O represents a hydrophobic amino acid (see Table 1).

Candidates for G-5-like structures are found in other GTPases. An analogous loop in the EF-Tu structure<sup>1</sup> is represented in several EF-Tu homologues by a conserved sequence, OOM(A/R/P)(G/T)SAL (Ser 173 in *Escherichia coli* EF-Tu). G protein  $\alpha$  chains contain a conserved sequence, T(C/Q)A(T/V)DT (Asp 368 in the  $\alpha$  chain of G<sub>s</sub>), which may have a similar role.

### Exchange of GTP for GDP

With  $K_d$  values between  $10^{-11}$  and  $10^{-7}$  M, GTPases bind guanine nucleotides far more tightly than necessary to ensure saturation by cytoplasmic concentrations of GTP ( $>10^{-4}$  M) or GDP ( $>10^{-5}$  M). GTPases are not engineered to respond to changes in concentration of either guanine nucleotide; instead,

FIG. 2 Structure of the GTP-bound form of p21<sup>ras</sup>. **a**, Cartoon of the protein's 3D structure, showing the bound guanine nucleotide and highlighting regions that interact with it: G-1 (orange), G-2 (blue), G-3 (red), G-4 (green), and G-5 (yellow). The cartoon, representing a truncated form of p21<sup>ras</sup> (residues 1–166)<sup>4</sup>, was drawn from coordinates kindly provided by E. F. Pai, A. Wittinghofer, and their associates, Max Planck Institut, Heidelberg. **b**, Selected amino acids that contact the bound GTP analogue Gpp(NH)p or that stabilize the guanine nucleotide binding site by interacting with residues in other regions (for example, Thr 144, Asn 116, and Lys 117). All contacts depicted in this panel, adapted from ref. 7, had hydrogen bond distances of 3.0 Å or less. Colours indicate the regions of p21<sup>ras</sup> (G-1 to G-5) in which each amino acid is located.





they cycle between GDP- and GTP-bound states, each of which must be sufficiently stable to prevent spontaneous exchange of one nucleotide for the other. Under certain circumstances, however, dissociation of GDP must be accelerated, to allow its replacement by GTP. We therefore find a fascinating collection of proteins (Table 2) that interact with GTPases to catalyse guanine nucleotide exchange.

The guanine nucleotide release proteins (GNRPs) act through the sequential mechanism shown in Fig. 3a. GNRP binds to the GDP-bound form of the GTPase ( $X \cdot \text{GDP}$ ), triggering dissociation of GDP. Although the GNRP·X complex is stable in the absence of guanine nucleotide, it is quite short-lived *in vitro* because GTP immediately enters the empty guanine nucleotide binding site. Then the GNRP dissociates from the transient GNRP·X·GTP complex. Both steps are reversible, with either GDP or GTP as the ligand. This sequential mechanism, in which the GTPase passes through a transient GNRP-bound 'empty' state, has been documented biochemically in the cases of EF-Tu<sup>10</sup> and G protein  $\alpha$  chains (reviewed in ref. 11), and is inferred from genetic experiments with yeast Ras proteins<sup>12</sup> and EF-Tu<sup>13</sup>.

For the GTPase cycle to work efficiently, GTP must replace GDP, rather than vice versa. The overall reaction may be driven in the forward direction by the excess of GTP over GDP in the cytoplasm, by greater affinity of the GTPase for GTP than for GDP, or by the intervention of other macromolecules, as described below.

**Regulation of guanine nucleotide exchange for elongation and initiation factors.** Guanine nucleotide exchange is subject to several different kinds of regulation (Table 2). In the case of EF-Ts, which catalyses GDP/GTP exchange for bacterial EF-Tu, the GNRP itself is constitutively active; no known ligands increase or decrease the activity of EF-Ts. To ensure that exchange goes in the forward direction (that GTP replaces GDP), the overall exchange reaction is regulated in the following way (see Fig. 3b): EF-Tu·GTP avidly binds aminoacyl-tRNA, whereas EF-Tu·GDP binds aminoacyl-tRNA weakly or not at all<sup>14</sup>. Consequently, by sequestering EF-Tu·GTP, aminoacyl-tRNA drives the exchange reaction forward<sup>10</sup>. If this sequestration did not occur, the equilibrium of the exchange reaction would favour EF-Tu·GDP, because EF-Tu has a higher affinity for GDP than for GTP.

In the absence of EF-Ts, EF-Tu binds GDP rather tightly ( $K_d = 2 \text{ nM}$ ). The GNRP accelerates replacement of GDP by GTP at a rate fast enough to maintain efficient protein synthesis. Guanine nucleotide exchange occurs without the intervention of a GNRP, however, in the case of EF-G, the bacterial elongation factor responsible for translocating peptidyl-tRNA from the A to the P site on the ribosome<sup>14</sup>. The intrinsic affinity of EF-G for GDP is low enough to allow its replacement by GTP. Why are GDP binding affinities of the elongation factors engineered in such a way that one requires a GNRP, while the other does not? The answer is unknown.

In the case of eukaryotic initiation factor 2, the GNRP is itself activated by binding GTP<sup>15</sup> (Table 2). The physiological role of this additional level of GTP-dependent regulation is unknown.

**Ligand-activated GNRPs for G proteins.** Cell-surface receptors for hormones and sensory stimuli transduce extracellular stimuli into cellular responses by promoting formation of the GTP-bound state of their GTPase targets, the heterotrimeric G proteins<sup>8,11,16-19</sup>. These ligand-regulated GNRPs (Table 2) comprise a rapidly growing family of proteins that share a characteristic topology (seven transmembrane regions) and conserved stretches of primary structure<sup>20,21</sup>.

The heterotrimeric structure of G proteins adds a useful element to the exchange reaction (Fig. 3c), that is, the  $\beta\gamma$  subunit, which has no counterpart in EF-Tu or monomeric GTPases like p21<sup>ras</sup>. The GDP-bound  $\alpha$  subunit of G proteins must bind to the  $\beta\gamma$  subunit before the  $\alpha\beta\gamma$ ·GDP complex can bind to the receptor, because the receptor binds the  $\alpha\beta\gamma$

heterotrimer much more tightly than it binds either  $\alpha$  or  $\beta\gamma$  alone<sup>22-24</sup>. This preference, combined with the preference of  $\beta\gamma$  for binding  $\alpha$ ·GDP rather than  $\alpha$ ·GTP, drives the exchange reaction in the direction of replacing GDP by GTP, rather than the other way around. The rapid dissociation of  $\beta\gamma$  from  $\alpha$ ·GTP (Fig. 3c) effectively prevents activated receptors from catalysing dissociation of GTP from  $\alpha$ . Thus  $\beta\gamma$  makes transmission of signals from the receptor to the G protein more efficient. Similarly, but by a different mechanism, aminoacyl-tRNA enhances efficiency of EF-Ts as a catalyst of guanine nucleotide exchange on EF-Tu.

An additional potential advantage of incorporating  $\beta\gamma$  into the GTPase cycle of G proteins stems from the fact that  $\beta\gamma$  binding stabilizes  $\alpha$ ·GDP—that is, GDP dissociates from  $\alpha\beta\gamma$  100-fold more slowly than from  $\alpha$  alone<sup>25</sup>. By reducing spontaneous (receptor-independent) release of GDP,  $\beta\gamma$  presumably damps signal transmission in the resting state, thereby increasing the ratio of signal to noise. The ability of  $\beta\gamma$  to enhance the signal-to-noise ratio of hormonal stimulation of adenylyl cyclase has been demonstrated *in vitro*<sup>26</sup>.

A short region of amino-acid sequence at the extreme N terminus of  $\alpha$  chains controls interactions with  $\beta\gamma$ <sup>27-29</sup>. This N-terminal sequence could interact directly with  $\beta\gamma$  or control the binding interaction indirectly.

**Molecular mechanism.** How do GNRPs promote the release of GDP? The G-4 and G-5 regions, which bind the guanine ring and interact with the G-1 region (Fig. 2b), comprise one obvious site at which binding of a GNRP might loosen bonds between

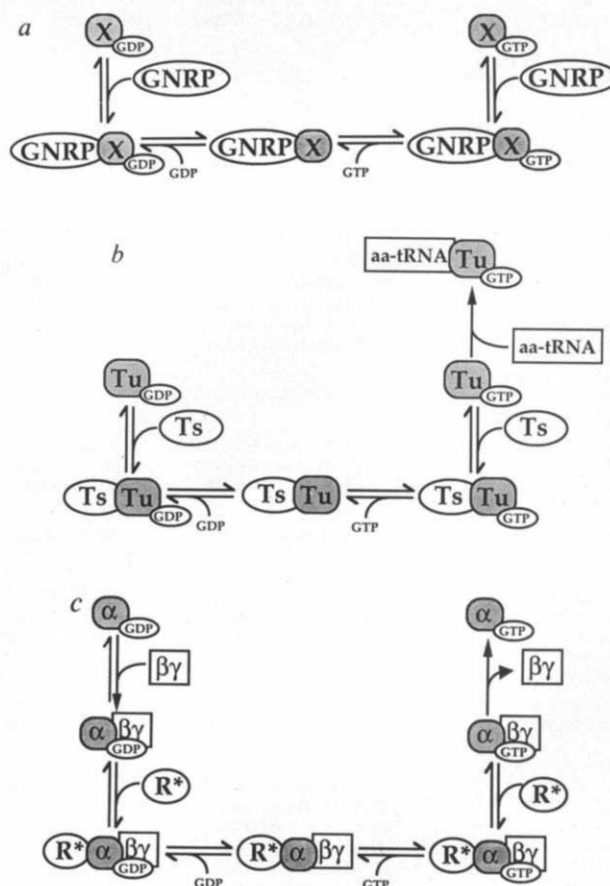


FIG. 3 Mechanisms for catalysis of guanine nucleotide exchange. *a*, Sequential catalysis of guanine nucleotide exchange on a generic GTPase (X) by an exchange protein (GNRP). *b*, The exchange reaction catalysed for EF-Tu by EF-Ts, in which aa-tRNA enhances the efficiency of GTP/GDP exchange. *c*, The exchange reaction catalysed for G protein  $\alpha$  chains by hormone receptors, in which  $\beta\gamma$  enhances efficiency of GTP/GDP exchange. See text for details.

TABLE 1 Sequence motifs in GTPase superfamily

| Consensus                                 | G-1<br>X0000GXGXGKS  | G-2<br>D-(X) <sub>n</sub> -T | G-3<br>QJ00DXAGJX | G-4<br>0000NKXD<br>TQ | Reference |
|-------------------------------------------|----------------------|------------------------------|-------------------|-----------------------|-----------|
| Elongation and initiation factors         |                      |                              |                   |                       |           |
| Elongation factors                        |                      |                              |                   |                       |           |
| <i>Prokaryotic</i>                        |                      |                              |                   |                       |           |
| EC EF-G                                   | 12 NigisAHiDaGKTTtt  | 51 DwmeqEreRGITitsa          | 84 iniiDtPGHv     | 138 iafvNKmD          | 65        |
| SR EF-G                                   | 12 NigiaAHiDaGKTTtt  | 51 DwmaqEreRGITItaa          | 84 iniiDtPGHv     | 138 iafiNKmD          | 66        |
| AN EF-G                                   | 12 NigiaAHiDaGKTTtt  | 51 DwmeqEreRGITItaa          | 77 vniiDtPGHv     | 131 ivfvNKmD          | 67        |
| EC LEPA                                   | 7 NfsiiAHiDhGKSTls   | 42 DsmdlEreRGITikaq          | 73 InfiDtPGHv     | 127 vpvlnKiD          | 68        |
| EC EFTU                                   | 13 NvgtiGHvDhGKTTlt  | 50 DnapeEkaRGITInts          | 76 yahvDcPGHa     | 131 ivf1NKcD          | 69        |
| TM EFTU                                   | 14 NvgtiGHvDhGKSTlt  | 51 DkapeEkaRGITInit          | 77 yahiDcPGHa     | 132 ivf1NKtD          | 70        |
| TT EFTU                                   | 14 NvgtiGHvDhGKTTlt  | 52 DkapeEraRGITInta          | 78 yshvDcPGHa     | 133 vvf1NKvD          | 71        |
| ML EFTU                                   | 14 NgtiGHvDhGKTTlt   | 53 DsapeErqRGITInis          | 79 yahvDaPGHa     | 134 lva1NKsD          | 72        |
| EG EFTU                                   | 14 NgtiGHvDhGKTTlt   | 51 DsapeEkaRGITInta          | 77 yahvDcPGHa     | 132 vvf1NKcD          | 73        |
| SC EFTU                                   | 50 NvgtiGHvDhGKTTlt  | 87 DkapeEraRGITista          | 113 yshvDcPGHa    | 131 vvf1NKvD          | 74        |
| MV EFTU                                   | 9 NvafiGHvDaGKTTtv   | 61 DglkeEreRGvTIdva          | 87 vtivDcPGHr     | 145 avavNKmD          | 75        |
| <i>Eukaryotic</i>                         |                      |                              |                   |                       |           |
| HS EF1A                                   | 9 NivviGHvDsGKTTtt   | 61 DklkaEreRGITidis          | 87 vtiiDaPGHr     | 149 ivgvNKmD          | 76        |
| SC EF1A                                   | 9 NvvviGHvDsGKTTtt   | 61 DklkaEreRGITidia          | 87 vtviDaPGHr     | 149 ivavNKmD          | 77        |
| LE EF1A                                   | 9 sivviGHvDsGKTTtt   | 61 DklkaEreRGITidia          | 87 ctviDaPGHr     | 149 icccNKmD          | 78        |
| HS GST1                                   | 76 NvvfiGHvDaGKTTtig | 128 DtnqeErdkGkTvevg         | 154 ftiiDaPGHk    | 216 ivliNKmD          | 79        |
| SC GST1                                   | 262 slifmGHvDaGKTTmg | 314 DtnkeErdndGkTievvg       | 340 ytiidDaPGHk   | 402 vvvvNKmD          | 80        |
| HAMSTER EF-2                              | 21 NmsviAHvDhGKTTlt  | 58 DtrkdEqrCITIkst           | 100 inliDsPGHv    | 154 v1mmNKmD          | 81        |
| DD EF-2                                   | 21 NmsviAHvDhGKTTls  | 58 scradEqrRGITikss          | 98 inliDsPGHv     | 152 v1fvNKvD          | 82        |
| Initiation factors                        |                      |                              |                   |                       |           |
| <i>Prokaryotic</i>                        |                      |                              |                   |                       |           |
| EC IF2                                    | 393 vvtimGHvDhGKTSll | 414 tkvasgeagGITqhig         | 440 itf1DtPGHa    | 494 vvavNKiD          | 83        |
| SF IF2                                    | 288 vvtimGHvDhGKTTll | 309 skvteqeagGITqhig         | 335 itf1DtPGHa    | 389 ivavNKiD          | 84        |
| <i>Unknown-prokaryotic</i>                |                      |                              |                   |                       |           |
| EC ERA                                    | 10 fiaivGrpnvGKSTll  |                              | 58 aiyvDtPGLh     | 120 ilavNKvD          | 33        |
| BS SPOOB                                  | 160 dvglvGfpsvGKSTll |                              | 208 fvmad1PGLi    | 278 iivaNKmD          | 85        |
| Small eukaryotic GTPases                  |                      |                              |                   |                       |           |
| Ras proteins                              |                      |                              |                   |                       |           |
| <i>Growth and differentiation</i>         |                      |                              |                   |                       |           |
| HS H/K/N-RAS                              | 5 K1VVVGgGGVGKSALT   | 32 YDPTIEDSY                 | 53 LDILDtAGQE     | 112 v1VGNKcD          | 86-88     |
| <i>Adenylyl cyclase regulation</i>        |                      |                              |                   |                       |           |
| SC RAS1                                   | 12 K1VVVGgGGVGKSALT  | 39 YDPTIEDSY                 | 60 LDILDtAGQE     | 119 vvVGNK1D          | 89        |
| SC RAS2                                   | 12 K1VVVGgGGVGKSALT  | 39 YDPTIEDSY                 | 60 LDILDtAGQE     | 119 vvVGNKsD          | 90        |
| <i>Unknown</i>                            |                      |                              |                   |                       |           |
| HS R-RAS                                  | 31 K1VVVGgGGVGKSALT  | 58 YDPTIEDSY                 | 79 LDILDtAGQE     | 138 v1VGNKaD          | 91        |
| DD RAS                                    | 5 K1V1VGgGGVGKSALT   | 32 YDPTIEDSY                 | 53 LDILDtAGQE     | 112 i1VGNKaD          | 92        |
| DM RAS2                                   | 7 K1VVVGgGGVGKSALT   | 34 YDPTIEDSY                 | 55 LDILDtAGQE     | 114 lmVGNKcD          | 93        |
| DM RAS1                                   | 5 K1VVVGpGGVGKSALT   | 32 YDPTIEDSY                 | 53 LDILDtAGQE     | 112 v1aGNKcD          | 94        |
| <i>Nutrient sensing/mating regulation</i> |                      |                              |                   |                       |           |
| SP RAS                                    | 10 K1VVVGdGGVGKSALT  | 37 YDPTIEDSY                 | 58 LDvLDtAGQE     | 117 v1VaNKcD          | 95        |
| <i>Ras antagonist?</i>                    |                      |                              |                   |                       |           |
| HS RAP1A                                  | 5 K1VVLGsGGVGKSALT   | 32 YDPTIEDSY                 | 53 LeILDtAGtE     | 112 i1VGNKcD          | 96        |
| HS RAP2                                   | 5 KvVVLGsGGVGKSALT   | 32 YDPTIEDSY                 | 53 LeILDtAGtE     | 112 i1VGNKvD          | 96        |
| SC RSR1                                   | 5 K1VVLGaGGVGKSALT   | 32 YDPTIEDSY                 | 53 LeILDtAGiA     | 112 v1iGNKaD          | 94        |
| <i>Unknown</i>                            |                      |                              |                   |                       |           |
| HS RAL                                    | 19 KvimvGsGGVGKSALT  | 46 YePTkaDSY                 | 67 LDILDtAGQE     | 126 11VGNKsD          | 97        |
| Rho family                                |                      |                              |                   |                       |           |
| HS RHOA                                   | 7 K1ViVGdGAcGKTCLL   | 34 YvPTVFeNY                 | 55 LaLWDtAGQE     | 113 i1VGNKkD          | 98        |
| HS RHOB                                   | 7 K1VvVGdGAcGKTCLL   | 34 YvPTVFeNY                 | 55 LaLWDtAGQE     | 113 i1LVANKkD         | 99        |
| SC RH01                                   | 12 K1ViVGdGAcGKTCLL  | 39 YvPTVFeNY                 | 60 LaLWDtAGQE     | 118 i1LVGKvD          | 100       |
| SC RH02                                   | 9 K1ViiVGdGAcGKTCLL  | 36 YhPTVFeNY                 | 57 LtLWDtAGQE     | 115 v1VGLKkD          | 100       |
| HS RAC                                    | 5 KcVvVGdGAvGKTCLL   | 32 YiPTVFaNY                 | 53 LgLWDtAGQE     | 111 i1LVGK1D          | 101       |
| <i>Cell polarity</i>                      |                      |                              |                   |                       |           |
| SC CDC42                                  | 5 KcVvVGdGAvGKTCLL   | 32 YvPTVFDNY                 | 53 LGLFDtAGQE     | 111 lvVGTqiD          | 102       |
| Vesicular transport                       |                      |                              |                   |                       |           |
| AT ARA                                    | 14 KivviGdsavGKSnl1  | 41 skaTiGveF                 | 63 aqiWDTAGQE     | 121 mlvGNKcD          | 103       |
| SP YPT3                                   | 12 K1lliGdsGVGKSnl1  | 39 sksTiGveF                 | 61 aqiWDTAGQE     | 119 mlvGNKtD          | 104       |
| HS RAB4                                   | 10 KflviGnagtGKScl1  | 37 snhTiGveF                 | 59 lqiWDTAGQE     | 117 ilcGNKkD          | 105       |
| HS RAB2                                   | 8 KyiiiGdtgVGKScl1   | 35 hdlTiGveF                 | 57 lqiWDTAGQE     | 115 mliGNKsD          | 105       |
| SC YPT1                                   | 10 K1lliGnsgVGKScl1  | 37 yisTiGvdF                 | 59 lqiWDTAGQE     | 117 llvGNKcD          | 106       |
| HS RAB1                                   | 13 K1lliGdsGVGKScl1  | 40 yisTiGvdF                 | 62 lqiWDTAGQE     | 120 llvGNKcD          | 105       |
| DD SAS1                                   | 17 K1lliGdsGVGKScl1  | 44 fitTiGidF                 | 66 lqiWDTAGQE     | 124 mliGNKcD          | 107       |
| SC SEC4                                   | 22 K1lliGdsGVGKScl1  | 49 fitTiGidF                 | 71 lqiWDTAGQE     | 129 llvGNKsD          | 108       |
| HS RAB3A                                  | 24 K1lliGnssVGKTSf1  | 51 fvsTvGidF                 | 73 lqiWDTAGQE     | 131 llvGNKcD          | 105       |
| HS RAB3B                                  | 24 K1lliGnssVGKTSf1  | 51 fvsTvGidF                 | 73 lqiWDTAGQE     | 131 i1vGNKcD          | 105       |
| HS RAB6                                   | 15 KlvlfGeqsVGKTSli  | 42 yqaTiGidF                 | 64 lqiWDTAGQE     | 122 mlvGNKtD          | 105       |
| BRL-RAS                                   | 4 KviiiGdsGVGKTSlm   | 31 ykaTiCadF                 | 53 mqiWDTAGQE     | 115 vv1GNKiD          | 109       |
| HS RAB5                                   | 22 K1vilGesavGKSslv  | 49 qesTiGaaf                 | 71 feiWDTAGQE     | 129 alsGNKaD          | 105       |



TABLE 1—continued

| Consensus                                        | G-1<br>X0000GXXGXGKS  | G-2<br>D-(X) <sub>n</sub> -T | G-3<br>QJ00DXAGJX | G-4<br>0000NKXD<br>TQ | Reference     |
|--------------------------------------------------|-----------------------|------------------------------|-------------------|-----------------------|---------------|
| ARF family                                       |                       |                              |                   |                       |               |
| ADP-ribosylation factors                         |                       |                              |                   |                       |               |
| HS ARF1                                          | 19 riLmvGLgaaGKTTil   | 45 tiPTigfnv                 | 63 ftvwDvGGqd     | 122 lvfaNKqD          | 110           |
| SC ARF1                                          | 19 riLmvGLggaGKTTvl   | 45 tiPTigfnv                 | 63 ftvwDvGGqd     | 122 lvfaNKqD          | 111           |
| Chromosome segregation                           |                       |                              |                   |                       |               |
| SC CIN4                                          | 18 roLilGLdmsGKSTiv   | 47 imPTvgfqi                 | 65 islwDiGGqr     | 127 invlNKiD          | 112           |
| Vesicular transport                              |                       |                              |                   |                       |               |
| SC SAR1                                          | 25 klLflGLdnaGKTTll   | 51 lqPTwhpts                 | 69 fttfDlGGhi     | 127 vilgNKiD          | 113           |
| G protein $\alpha$ -subunits—signal transduction |                       |                              |                   |                       |               |
| Multicellular eukaryotes                         |                       |                              |                   |                       |               |
| GS                                               | 42 rLLLLGAGESGKSTiv   | 196 DILrcRvltTsGfE           | 219 FhmFDVGGQR    | 288 iLFLNKqD          | 114           |
| GO                                               | 35 KLLL GAGESGKSTiv   | 174 DvLrtRvkTtGivE           | 197 FkmFDVGGQR    | 266 iLFLNKkD          | 115           |
| GI                                               | 35 KLLL GAGESGKSTiv   | 173 DiLrtRvkTtGivE           | 196 FrlFDVGGQR    | 265 iLFLNKkD          | 115           |
| GT                                               | 31 KLLL GAGESGKSTiv   | 169 DvLrsRvkTtGiE            | 192 FrmFDVGGQR    | 261 vLFLNKkD          | 116           |
| HS GZ                                            | 35 KLLL GtsnSGKSTiv   | 174 DiLrsRdmTtGivE           | 197 FkmFDVGGQR    | 266 iLFLNKkD          | 117, 118      |
| <i>Saccharomyces cerevisiae</i>                  |                       |                              |                   |                       |               |
| Mating regulation                                |                       |                              |                   |                       |               |
| SC GPA1                                          | 43 KLLL GAGESGKSTvl   | 292 DiLkgRikTTGITE           | 315 FkvLDaGGQR    | 384 iLFLNKiD          | 119, 120      |
| Unknown                                          |                       |                              |                   |                       |               |
| SC GPA2                                          | 125 KvLLL GAGESGKSTvl | 268 DiLrsRqmTSGIFD           | 292 mhiyDVGGQR    | 361 vLFLNKiD          | 121           |
| <i>Dictyostelium discoideum</i>                  |                       |                              |                   |                       |               |
| DD DG1                                           | 38 KLLL GAGESGKSTia   | 175 DvLrsRtkTTGIE            | 198 FrmFDVGGQR    | 267 iLFLNKrD          | 122           |
| DD DG2                                           | 33 KLLL GAGESGKSTis   | 177 DiLhtRvmTRGVHE           | 200 FrlvDVGGQR    | 268 iLFLNKsD          | 122           |
| Protein translocation across membranes           |                       |                              |                   |                       |               |
| MM SRP54                                         | 102 vimfvGlgGsGKTTtc  | 138 DTFRAGA                  | 186 iiivDTsGRh    | 244 svivTKlD          | 123, 124      |
| SC SRP                                           | 102 vimmvGlgGsGKTTtc  | 138 DTFRAGA                  | 186 viivDTsGRh    | 244 aviiTKlD          | 125           |
| SP SRP                                           | 110 iimfvGlgGsGKTTsc  | 146 DTFRAGA                  | 194 iiivDTsGRh    | 252 aiilTKlD          | 125           |
| EC FFH                                           | 101 vvlmaGlgGaGKTTsv  |                              | 186 vllvDTaGRl    | 244 gvvlTKvD          | 123, 124, 126 |
| EC FTSY                                          | 294 vilmvGvnGvGKTTti  | 330 DTFRAAA                  | 378 vliaDTaGRl    | 442 gitlTKlD          | 127           |
| DOG SR $\alpha$                                  | 419 vvtfcGvnGvGKSTnl  | 455 DTFRAGA                  | 516 vvlvDTaGRm    | 584 givlTKfd          | 128           |

The table shows the amino-acid sequences of putative regions G1–G4 (as defined in the text) in one or more representatives of each GTPase family, plus a consensus sequence for the family. Assignments of individual GTPases to families were made primarily on structural rather than functional grounds, with the result that some proteins are grouped in a particular functional category without supporting biochemical evidence. Bold type indicates residues conserved in nearly all GTPases; the other upper-case letters indicate amino-acid identities used to align sequences and assign proteins to a particular family. Amino acids are designated according to the single-letter code: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. In consensus sequences, X indicates any amino acid, whereas O and J represent, respectively, hydrophobic and hydrophilic residues. Species designations are: EC, *E. coli*; SR, *Spirulina platensis*; AN, *Anacystis nidulans*; TM, *Thermotoga maritima*; TT, *Thermus thermophilus*; ML, *Micrococcus luteus*; EG, *Euglena gracilis*; SC, *Saccharomyces cerevisiae*; HS, *Homo sapiens*; LE, *Lycopersicon esculentum*; DD, *Dictyostelium discoideum*; SF, *Streptococcus faecium*; BS, *Bacillus subtilis*; DM, *Drosophila melanogaster*; SP, *Schizosaccharomyces pombe*; AT, *Arabidopsis thaliana*; MM, *Mus musculus*.

the GTPase and the bound nucleotide. Genetic, biochemical, and immunological probes suggest that GNRPs for G protein  $\alpha$  chains and EF-Tu do interact with these regions, and also (in the  $\alpha$  chains) with a predicted C-terminal  $\alpha$  helix<sup>30</sup> that may be homologous to the C-terminal  $\alpha 5$  helix (residues 152–166) in crystals of the truncated form of p21<sup>ras</sup> (Fig. 2a). This evidence, summarized in Table 3, predicts that GNRPs will in general be found to bind to GTPase regions that correspond to a topographically discrete portion of the p21<sup>ras</sup> structure, corresponding to the top and upper right faces of the protein as it is depicted in Fig. 2a. Figure 4 schematically depicts probable sites for GNP interactions with EF-Tu and G protein  $\alpha$  chains (panel a).

Through these interactions GNRPs probably catalyse dissociation of GDP by relaxing bonds (Fig. 2b) between the guanine ring and side chains of amino acids in region G-4 and between G-4 residues and side chains of amino acids in regions G-1 and G-5. This idea is in keeping with abundant evidence (Table 3) that mutational replacements of amino acids in both these regions reduce the affinity with which GTPases bind GDP. Indeed, one such mutation in the G-4 region of EF-Tu seems to reduce guanine nucleotide binding affinity so drastically that the mutant GTPase freezes in a conformation that binds tightly to its GNP, EF-Ts (Table 3, and ref. 13).

Mutations in the G-4 and G-5 regions markedly reduce the binding affinities of Ras proteins for bound nucleotide (Table 3). Accordingly, we propose (Fig. 4b) that GNRPs may act on these regions of Ras proteins to release GDP. No biochemical evidence, however, points to these (or any other) portions of the Ras proteins as a target for GNRPs. Equally plausible targets include portions of the proteins that interact with phosphate groups of the nucleotide or with the critically important Mg<sup>2+</sup> ion—that is, residues in region G-1, Asp 57 in G-3, etc.

### GTP-vs GDP-bound forms of p21<sup>ras</sup>

Crystal structures of the p21<sup>ras</sup>·GDP complex<sup>3,5,31</sup> have been compared to structures of the protein bound to GTP itself<sup>31</sup> or to two GTP analogues, Gpp(NH)p<sup>7</sup> and Gpp(CH<sub>2</sub>)p<sup>5</sup>. All the comparisons reveal prominent GTP-induced changes in two regions of the protein: the G-2 loop (amino acids 32–38) and the G-3 loop together with the  $\alpha 2$  helix just downstream (specifically, residues 60–76). These regions are located close to one another and exposed on the outer surface of the molecule—at the bottom and front of the structure as depicted in Fig. 2a.

How does GTP induce the conformational change? A dramatic change in the orientation of Thr 35 probably accounts for the change in G-2. The side chain of this residue points away

TABLE 2 Guanine nucleotide release proteins

| Protein                                  | GTPase target           | Regulated by                            | Organism                                |
|------------------------------------------|-------------------------|-----------------------------------------|-----------------------------------------|
| EF-Ts                                    | EF-Tu                   | End-product (EF-Tu·GTP)                 | <i>E. coli</i> <sup>10</sup>            |
| Guanine nucleotide exchange factor (GEF) | eIF-2                   | GTP binding to GEF                      | Rabbit <sup>15</sup>                    |
| CDC25                                    | RAS1, RAS2              | Unknown                                 | <i>S. cerevisiae</i> <sup>129-131</sup> |
| Hormone and sensory receptors            | G protein heterotrimers | Hormones, pheromones, photons, odorants | Eukaryotes <sup>11,16-18</sup>          |

from the bound nucleotide in the GDP structure, but flips toward the nucleotide in the GTP structure, where it interacts directly with both the catalytic  $Mg^{2+}$  and the  $\gamma$ -phosphate of GTP (Fig. 2b). This GTP-induced change in orientation of Thr 35 may be propagated to adjacent G-2 residues. Conformational change in the G-3 loop and downstream  $\alpha$  helix may be similarly induced by the interaction between the  $\gamma$ -phosphate and Gly 60 (Fig. 2b). The glycine residue's inherent flexibility allows it to rotate significantly with respect to the preceding  $\beta$  strand ( $\beta$ 3, Fig. 2a); this rotation is probably necessary for the conformational change in the 16 residues downstream, which alters the orientation and length of helix  $\alpha$ 2. Not surprisingly, both Thr 35 and Gly 60 are conserved throughout the superfamily of GTPases (Table 1).

Even before the crystal structures became available, phenotypes produced by mutations in  $p21^{ras}$  had already implicated both regions as sites of conformational change. Many mutations in these regions prevent neoplastic transformation by  $p21^{ras}$  and also prevent GTP-dependent interaction of  $p21^{ras}$  with the GTPase activating protein, GAP; Table 4 summarizes these observations.

### GTP hydrolysis by $p21^{ras}$

The crucial biochemical importance of the GTPase reaction, combined with its potential as a target for useful drugs, has inspired many experiments. A plausible molecular mechanism, consistent with a large body of observations, has recently been proposed<sup>7</sup>: the carbamoyl oxygen of Gln 61 activates a water molecule (Wat 175 in the high-resolution structure<sup>7</sup>), which mounts a nucleophilic attack on the  $\gamma$ -phosphorus of GTP. The GTPase reaction is thought to follow an associative in-line reaction pathway, with inversion of configuration at the  $\gamma$ -phosphate<sup>32</sup>. Such a pathway requires that the attacking water molecule be located directly opposite the leaving group (the  $\beta$ -phosphate)—this is precisely the location of Wat 175 (ref. 7).

The side chain of Gln 61 is in the right place to activate Wat 175, in crystals of both the GTP<sup>31</sup> and Gpp(NH)p-bound<sup>7</sup> forms of  $p21^{ras}$ . It should be noted, however, that these crystals show more than one orientation for Gln 61 and other residues in the G-3 loop<sup>7</sup>, suggesting that this region in the GTP-bound protein can flip between at least two conformations; only one of the two detected orientations of Gln 61 is consistent with the proposed GTPase reaction mechanism.

The GTPase-triggering role proposed for Gln 61 is consistent with the conservation of glutamine at this position in many other GTPases, with crystal structures of mutant  $p21^{ras}$  molecules, and with a large body of genetic evidence, including oncogenic effects of mutations of this residue in  $p21^{ras}$  and G protein  $\alpha$  chains (see Table 5). It is thus surprising to find—in view of the strict conservation of aspartates and glycines at positions corresponding to residues 57 and 60 of  $p21^{ras}$ —that many GTPases contain amino acids other than glutamine at the position corresponding to residue 61 (Table 1). The ERA protein of *E. coli* and the RSR1 protein of *Saccharomyces cerevisiae* contain, respectively, leucine<sup>33</sup> and isoleucine<sup>34</sup> at this position; it is difficult to imagine the uncharged side chains of these residues performing a role similar to that proposed for Gln 61 of  $p21^{ras}$ . Although further experiments may firmly establish the role of this glutamine in GTP hydrolysis by  $p21^{ras}$  and G protein  $\alpha$  chains, it is likely that many other GTPases use a different catalytic mechanism.

Like mutations of the Gln 61 codon, mutations in the G-1

region of  $p21^{ras}$  inhibit GTPase activity and contribute to malignant transformation; of the codons in this region, Gly 12 is most often mutated in human tumours<sup>35</sup>. Because binding of GTP vs GDP does not alter the position and orientation of Gly 12<sup>3-5</sup>, this residue is not likely to participate directly in GTP hydrolysis. Instead, crystal structures of mutant  $p21^{ras}$  show<sup>36</sup> that the side chains of Arg 12 or Val 12 inhibit GTP hydrolysis by occupying space: the arginine side chain displaces the postulated catalytic water molecule, Wat 175, whereas the valine side chain pushes Gln 61 and Wat 175 far enough apart to prevent the GTPase reaction.

With a single class of exceptions, to be described later, purified GTPases hydrolyse GTP at extraordinarily slow rates *in vitro*, and very much faster in the presence of appropriate GAP molecules. The  $k_{cat-GTP}$  of  $p21^{ras}$ , for instance, is  $\sim 0.02 \text{ min}^{-1}$  in the absence of GAP<sup>37</sup>, and GAP increases the rate many fold. Similarly, the mRNA-programmed ribosome strikingly increases the  $k_{cat-GTP}$  of EF-Tu<sup>14</sup>. Why do the isolated proteins hydrolyse GTP so slowly? How does GAP (or the ribosome) accelerate the reaction?

One explanation for the intrinsically low  $k_{cat-GTP}$  is to imagine that Gln 61 can take on many different orientations in  $p21^{ras}$ , and that only one of these orientations enables it to activate Wat 175. This view is in keeping with the multiple orientations of Gln 61 seen in the high-resolution structure<sup>7</sup>. According to this view, GAP acts simply to stabilize the conformation of the *ras* protein most favourable for GTP hydrolysis. An alternative explanation is that binding of GTP induces a slow conformational change that manoeuvres Gln 61 into an orientation favourable for GTP hydrolysis. This explanation seems less likely, although derivatives of GTP or Gpp(NH)p seemed to show an appropriately slow change in fluorescence following binding to  $p21^{ras}$ <sup>38</sup>. Time-resolved X-ray crystallographic study of the GTP-bound protein<sup>31</sup> provided no evidence for such a slow conformational change, however, even though  $p21^{ras}$  in the crystals does hydrolyse GTP at the same (slow) rate seen in solution<sup>31,39</sup>.

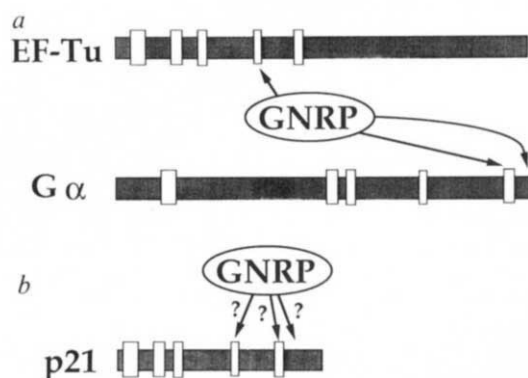


FIG. 4 Probable sites of interaction between exchange proteins (GNRP) and EF-Tu or G protein  $\alpha$  subunits (a) and postulated interaction between GNRP and  $p21^{ras}$  (b). Each shaded bar represents the linear sequences of a designated GTPase (N terminus on the left); the unfilled boxes indicate locations of regions G-1 to G-5, which participate in binding and hydrolysis of GTP (see Fig. 1 and Table 1). Arrows in a point to locations where evidence indicates interaction with the appropriate GNRP; this evidence is summarized in Table 3 and discussed in the text. Arrows in b indicate sites at which the GNRP for  $p21^{ras}$  may interact (see text and Table 3).



TABLE 3 GTPase regions that interact with GNRPs

| GTPase                                            | Region                     | Experiment                                                                                                       | Result                                                                                                                                                                                                                                         |
|---------------------------------------------------|----------------------------|------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| $\alpha_s$                                        | Predicted C-terminal helix | Mutation replacing Arg 389 by proline                                                                            | Prevents $\beta$ -adrenergic and PGE <sub>2</sub> stimulation of adenyl cyclase <sup>132</sup>                                                                                                                                                 |
| GPA/SGC1<br>( $G\alpha$ in <i>S. cerevisiae</i> ) | Predicted C-terminal helix | Mutational removal of five carboxy terminal residues                                                             | Sterile phenotype (uncoupling of G protein from pheromone receptor) <sup>133</sup>                                                                                                                                                             |
| $\alpha_t$                                        | G-5                        | Peptide (residues 311–329)                                                                                       | Both peptides inhibit photorhodopsin-stimulated GTPase and transducin-induced M-II conformation of rhodopsin <sup>134</sup>                                                                                                                    |
|                                                   | Predicted C-terminal helix | Peptide (residues 340–350)                                                                                       |                                                                                                                                                                                                                                                |
|                                                   | G-5                        | Monoclonal antibody against residues 311–329                                                                     | Inhibits transducin-induced M-II conformation of rhodopsin and light stimulation of cGMP phosphodiesterase <sup>135,136</sup>                                                                                                                  |
|                                                   | Predicted C-terminal helix | Cloning of arrestin, which competes with transducin for binding to photorhodopsin                                | Similar C-terminal sequences of arrestin and transducin may participate in binding to photorhodopsin <sup>137</sup>                                                                                                                            |
| $\alpha_i, \alpha_o, \alpha_t$                    | Predicted C-terminal helix | Pertussis toxin-catalysed attachment of ADP-ribose to conserved cysteine (Cys 347 of $\alpha_t$ ) <sup>138</sup> | Prevents ligand- or light-induced stimulation of GTPase, effector activation <sup>11,17–19</sup>                                                                                                                                               |
| EF-Tu                                             | G-4                        | Binding of EF-Ts to EF-Tu                                                                                        | Blocks alkylation of Cys 137 in EF-Tu <sup>139</sup><br>Dominant negative inhibition of protein synthesis, presumably by reducing affinity for binding GTP*, thereby producing a mutant EF-Tu that binds up most available EF-Ts <sup>13</sup> |
|                                                   | G-4                        | Mutational replacement of Lys 136 by glutamate or glutamine                                                      |                                                                                                                                                                                                                                                |
| RAS2                                              | G-5                        | Mutational replacement of Thr 152 by isoleucine                                                                  | Bypasses requirement for putative GNRp, CDC25 <sup>140</sup>                                                                                                                                                                                   |
| p21 <sup>ras</sup>                                | G-4                        | Mutations that replace amino acids, decreasing affinity for GDP                                                  | The same mutations promote neoplastic transformation, probably by bypassing requirement for a GNRp <sup>141</sup>                                                                                                                              |

\* This paper<sup>13</sup> quite reasonably suggests that Lys 136 is not a site of EF-Ts binding, because its replacement by isoleucine appears to stabilize, rather than prevent, binding of EF-Ts. Although the lysine itself probably does not interact with the exchange protein, side chains of other residues in this loop may do so.

Because GAP and the ribosome bind preferentially to the GTP-bound forms of their respective GTPases, it is reasonable to imagine that they bind to a region whose conformation is altered by binding GTP—for example, in p21<sup>ras</sup>, to the G-2 and G-3 regions (Fig. 2a)<sup>4,5</sup>. Table 6 summarizes abundant evidence from studies of mutant phenotypes, synthetic peptides and antibodies showing that the G-2 region is involved in GAP-mediated stimulation of GTPase activity. Binding to loop G-2 and region G-3 would provide obvious opportunities for GAP to nudge bound GTP and key catalytic residues (Thr 35, Gln 61) into positions that favour GTP hydrolysis. In addition, or alternatively, GAP could insert an extrinsic catalytic group, perhaps even the essential nucleophile, into the guanine nucleotide binding site; after all, the crystal structures show the  $\gamma$ -phosphate of GTP exposed to the ambient medium through a 'window' between regions G-2 and G-3, exactly where GAP might be expected to bind (see, for example, Fig. 8 in ref. 5).

### Similarities between p21<sup>ras</sup> EF-Tu and $\alpha$ chains

Although crystal structures of GTP-bound EF-Tu and G protein  $\alpha$  chains are not available, mutations and biochemical analysis implicate region G-3 and sequences immediately downstream as sites of GTP-induced conformational change in these GTPases also (see Table 4). In both EF-Tu and the  $\alpha$  chain of  $G_s$  ( $\alpha_s$ ), for example, replacement by alanine of glycine residues cognate to Gly 60 of p21<sup>ras</sup>—that is, attachment of a mere methyl group, but at a pivotal location—prevents bound GTP from changing the protein's conformation<sup>40,41</sup>. Similarly, binding to GTP prevents trypsin from cleaving G protein  $\alpha$  chains at a topographically cognate to the  $\alpha$ 2 helix of p21<sup>ras</sup>, just downstream from region G-3<sup>22,40,42</sup>.

Effects of cognate mutations in p21<sup>ras</sup> and  $\alpha_s$  indicate that the two proteins use parallel molecular mechanisms and structural elements in GTP hydrolysis. Thus mutational replacement

of Gln 227 in  $\alpha_s$  profoundly inhibits GTP hydrolysis<sup>43,44</sup> and contributes to growth of human tumours<sup>45</sup>, just like replacements of the cognate Gln 61 in p21<sup>ras</sup> (refs 35, 46). Similarly, mutational replacement of glycines at positions 49 and 12 of  $\alpha_s$  and p21<sup>ras</sup>, respectively, both inhibit GTP hydrolysis also<sup>35,43,44</sup>.

### Differences between p21<sup>ras</sup> and $\alpha$ chains

Unlike conditional GTPases like p21<sup>ras</sup> or EF-Tu, the intrinsic  $k_{cat-GTP}$  of the  $\alpha$  chain of  $G_s$  is very much larger (for example, 3–5 compared with 0.02 min<sup>-1</sup> for  $\alpha_s$  and p21<sup>ras</sup>, respectively). Moreover, this faster rate of GTP hydrolysis does not require the presence of a GAP-like protein. In the case of  $\alpha_s$  the  $k_{cat-GTP}$  value determined for the pure protein<sup>43</sup> is remarkably close to the value determined indirectly in cell membranes<sup>44,45</sup>. In this respect the G protein  $\alpha$  chains are unique among the known GTPases. What is the molecular basis of the difference between them and the conditional GTPases?

Figure 5 depicts a highly speculative but testable hypothesis: A built-in GAP-like domain of G protein  $\alpha$  chains maintains constitutive GTPase activity by interacting with the G-2 and G-3 regions. The postulated<sup>45,47</sup> GAP-like domain, 110–130 residues long in different  $\alpha$  chains, is inserted between the G-1 and G-2 regions of the guanine nucleotide binding domain (Table 1), presumably at a site topologically analogous to the junction between  $\alpha$ 1 and loop 2 of p21<sup>ras</sup> (see Fig. 2a). This insertion appears only in the family of heterotrimeric G proteins, and never in conditional GTPases. The putative  $\alpha$  chain G-2 region acted upon by the built-in GAP-like domain is located around a threonine residue (Thr 204 in  $\alpha_s$ ; see Table 1) cognate to Thr 35 of p21<sup>ras</sup>; this is the only invariant threonine residue found between the G-1 and G-3 regions of  $\alpha$  chains.

Recently discovered oncogenic mutations in  $\alpha$ -chain genes<sup>45,48</sup> highlight a second unique feature of this class of GTPases—an invariant arginine residue essential in maintaining

TABLE 4 Correlates of GTP-induced conformational change in different GTPases

| GTPase                         | Region                                                                            | Experiment                                                                         | Observation                                                                                                                                                                                                                                        | Interpretation                                                                                                       |
|--------------------------------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|
| p21 <sup>ras</sup>             | G-2                                                                               | Point mutations that replace amino acids                                           | Prevent neoplastic transformation <sup>96,142,143</sup>                                                                                                                                                                                            | Region may interact with 'effector'                                                                                  |
|                                |                                                                                   |                                                                                    | Prevent GTP-dependent interaction with GAP <sup>56,57,142,144,145</sup>                                                                                                                                                                            | Region interacts with GAP                                                                                            |
|                                | G-3                                                                               | Nuclear magnetic resonance                                                         | Resonances originating from amino acids in G-2 and $\beta$ 3 differ in GDP- and GTP-bound forms <sup>146</sup>                                                                                                                                     | GTP binding changes relative orientations of G-2 region and $\beta$ 3                                                |
|                                |                                                                                   | Small deletion (e.g., residues 64–68)                                              | Inactivates oncogenic p21 <sup>ras</sup> <sup>61</sup>                                                                                                                                                                                             | Deletion either prevents the conformational change or prevents it from being transmitted to effector                 |
| $\alpha_s, \alpha_o, \alpha_t$ | Region equivalent to helix $\alpha_2$ of p21 <sup>ras</sup> , downstream from G-3 | Exposure to trypsin                                                                | GTP analogs prevent tryptic cleavage at position corresponding to arginine-231 of $\alpha_s$ <sup>22,40,42</sup>                                                                                                                                   | GTP changes conformation of predicted $\alpha$ helix <sup>30</sup> corresponding to $\alpha_2$ to p21 <sup>ras</sup> |
| $\alpha_s$                     | G-3                                                                               | Replacement by alanine of Gly 226 (corresponding to Gly 60 of p21 <sup>ras</sup> ) | Without preventing binding of GTP, this mutation inhibits GTP-dependent dissociation from $\beta\gamma$ and stimulation of adenyl cyclase; it also prevents GTP analogs from protecting the protein from tryptic cleavage at Arg 231 <sup>40</sup> | Addition of methyl group to conserved glycine residue prevents GTP-induced conformational change                     |
| EF-Tu                          | G-3                                                                               | Replacement by alanine of Gly 83 (corresponding to Gly 60 of p21 <sup>ras</sup> )  | Prevents GTP-dependent association with aminoacyl-tRNA <sup>41</sup>                                                                                                                                                                               | Addition of methyl group to conserved glycine residue prevents GTP-induced conformational change                     |

constitutive GTP hydrolysis. This residue (Arg 201 in  $\alpha_s$ ) is located in region G-2, only three residues upstream from the threonine we propose to correspond to Thr 35 of p21<sup>ras</sup> (see  $\alpha$  chain G-2 regions in Table 1).

Cholera toxin-catalysed attachment of ADP-ribose to this arginine<sup>49</sup> markedly slows GTP hydrolysis by  $\alpha_s$ <sup>50</sup> and  $\alpha_t$ <sup>51</sup>. The GTPase-inhibiting effect of this covalent modification is mimicked by oncogenic (*gsp*) mutations of  $\alpha_s$ , found in pituitary and thyroid tumours<sup>45,48</sup>, which replace Arg 201 with other amino acids, including cysteine or histidine. Deliberate replacements of Arg 201 by lysine, glutamate or alanine produce the same inhibition of GTPase<sup>52</sup>. In addition to the *gsp* mutations<sup>45</sup>, mutations (termed *gip2*) that replace the cognate arginine residue in a second  $\alpha$  chain,  $\alpha_{i2}$ , have been found in tumours of human adrenal cortex and ovary<sup>48</sup>. Taken together, these observations imply that the side chain of this conserved arginine residue is essential in triggering GTP hydrolysis by  $\alpha_s$ ,  $\alpha_{i2}$  and  $\alpha_t$ —and probably by all G protein  $\alpha$  chains.

How might the side chain of this arginine promote GTP hydrolysis? Although it cannot serve as a nucleophile, it could perhaps help to manoeuvre a different side chain into a position favourable for nucleophilic attack on the  $\gamma$ -phosphorus of GTP. If the proposed location of G-2 in the  $\alpha$  chains is correct, Tyr 32 of p21<sup>ras</sup> would be cognate to Arg 201. Although the side chains of tyrosine and arginine are unlikely to perform identical facilitating roles in the GTPase reactions of their respective proteins, it may be significant that the position and orientation of Tyr 32 differ markedly in the GDP and GTP forms of p21<sup>ras</sup>; binding of GTP causes the tyrosine side chain to swing outward, partially blocking the entrance to the guanine nucleotide binding pocket<sup>5</sup>. GTP may induce a similarly drastic change in the position of the corresponding arginine of  $\alpha$  chains.

It is worth noting that the elongation and initiation factors, as well as a number of other GTPases, also contain a conserved arginine three residues upstream from the conserved threonine of region G-2 (Table 1). Studies of site-directed mutations could determine whether replacements of Arg 58 and Thr 61 in EF-Tu affect hydrolysis of GTP or GTP-dependent binding to amino acid-tRNA or the programmed ribosome.

### GTPase interactions with effectors

Because the cellular functions of GTPases are determined by their GTP-dependent affinities for binding other macromole-

cules, it is of considerable interest to identify regions of the GTPases that interact specifically with effector molecules—with aminoacyl-tRNA and the programmed ribosome in the case of EF-Tu, with enzymes and ion channels in the case of G protein  $\alpha$  chains, and with GAP or other as yet unidentified proteins in the case of p21<sup>ras</sup>. For EF-Tu, unfortunately, little or no firm evidence points to portions of this multi-domain protein that interact with either aminoacyl-tRNA or the ribosome. Several observations, described below, suggest that G protein  $\alpha$  subunits and p21<sup>ras</sup> have evolved different ways of interacting with their effectors.

Adenyl cyclase is the only effector target of the G proteins so far tested. The first observations<sup>53</sup> were made with chimaeric G protein  $\alpha$  chains in which N-terminal sequences (60% of the chimaera) were derived from  $\alpha$  chains of G proteins that do not stimulate adenyl cyclase (that is,  $G_{i2}$  and  $G_{i1}$ ). These N-terminal portions contain the postulated GAP-like domain of the  $\alpha$  chain, as well as the putative G-2 and G-3 regions whose conformations in p21<sup>ras</sup> change on binding of GTP.

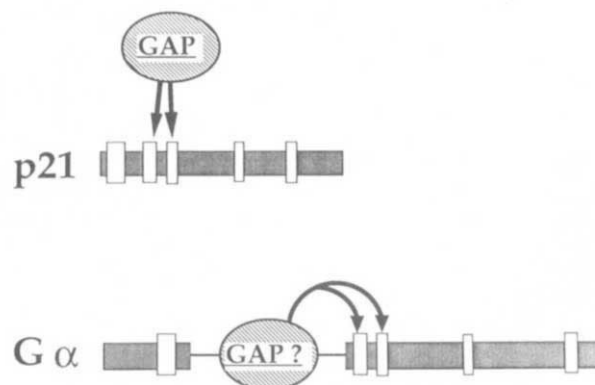


FIG. 5 Proposed roles for GAP or a putative GAP-like domain in stimulating GTP hydrolysis. Shaded bars represent the linear sequences of p21<sup>ras</sup> or G protein  $\alpha$  chains, with white boxes indicating the locations of guanine nucleotide site regions G-1 to G-5. GAP and the GAP-like domain are proposed to bind to cognate regions of their respective GTPases, including regions G-2 and G-3.



TABLE 5 Role of Gln 61 and cognate residues in GTP hydrolysis in other proteins

| GTPase                    | Experimental observation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | Interpretation                                                                                                                                                                                                                                                                                                                                               |
|---------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| p21 <sup>ras</sup>        | Somatic mutations that replace Gln 61 reduce GTPase and contribute to generation of human tumours <sup>35</sup><br>Replacement of Gln 61 by 17 other amino acids promotes neoplastic transformation, whereas replacement by glutamate does not <sup>46</sup><br><br>Crystals of His 61 and Leu 61 mutants show that these residues could not activate Wat 175, near the $\gamma$ -phosphate of GTP; similarly, crystals of Arg 12 and Val 12 mutants show how these mutations prevent access of Gln 61 to Wat 175 <sup>36</sup> | Glutamine side chain is involved in GTP hydrolysis<br><br>Glutamine side chain is involved in GTP hydrolysis; glutamate may be able to maintain GTPase because it possesses an identically positioned carbonyl group<br>Glutamine is conserved at position 61 because of its specific capacity to activate nucleophilic attack by a catalytic water molecule |
| Other small GTPases       | Glutamine highly conserved in all except the Rap proteins (see Table 1)                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Glutamine side chain is involved in GTP hydrolysis                                                                                                                                                                                                                                                                                                           |
| Rap-1A                    | Threonine is the natural amino acid at position 61; its replacement by glutamine increases GTPase to level found in p21 <sup>ras</sup> and increases sensitivity of Rap-1A GTPase to stimulation by GAP <sup>147</sup>                                                                                                                                                                                                                                                                                                          | For GTP hydrolysis, the glutamine side chain is better than that of threonine                                                                                                                                                                                                                                                                                |
| EF-Tu                     | Histidine is located at the position corresponding to Gln 61 of p21 <sup>ras</sup> ; its replacement by glycine reduces GTPase activity more than 10-fold (R. H. Cod and A. Parmeggiani, cited in ref. 7)                                                                                                                                                                                                                                                                                                                       | Side chain of histidine may have a function similar to that of glutamine                                                                                                                                                                                                                                                                                     |
| G protein $\alpha$ chains | Somatic mutations replace cognate glutamine of $\alpha_s$ and $\alpha_{12}$ by other residues in human tumours, constitutively activating the proteins and reducing GTPase activity <sup>45,48</sup>                                                                                                                                                                                                                                                                                                                            | Glutamine side chain is involved in GTP hydrolysis in G proteins                                                                                                                                                                                                                                                                                             |

Chimaeric proteins containing only the C-terminal 40% of  $\alpha_s$  unequivocally mediated GTP-dependent stimulation of cyclic AMP synthesis<sup>53</sup>. This C-terminal portion of  $\alpha_s$  apparently contains the structural determinants required for GTP-triggered interaction with the effector.

These initial observations leave several important questions unanswered. Within the C-terminal 40% of  $\alpha_s$ , which regions carry information required for specific interaction with adenyl cyclase? Experiments with another  $\alpha_s/\alpha_{12}$  chimaera<sup>54</sup> show that the 36 residues of  $\alpha_s$  at its extreme C terminus do not carry such information. Additional chimaeras will identify the effector binding region more precisely. Can these observations be generalized to other G protein  $\alpha$  chains and other effectors? Parallel experiments in other signalling pathways are needed to answer this question.

Finally, we will want to know how the  $\alpha$  chain propagates a conformational change from residues in regions G-2 and G-3, which contact the  $\gamma$ -phosphate of GTP, to regions located in the protein's C-terminal 40%. Could the 'extra' domain of these proteins, postulated to serve a GAP-like role, also participate in propagating a GTP-induced change in conformation? In support of this notion, a structural change in the 'extra' domain of  $\alpha_0$  (alkylation of a cysteine) does induce a functional change in the protein's extreme C terminus, decreased susceptibility to ADP-ribosylation by pertussis toxin<sup>55</sup>. Here p21<sup>ras</sup> offers few clues, because the crystal structures reveal no GTP-induced

change in the position of any amino acid distal to residue 76, and because the conformation of GAP-bound p21<sup>ras</sup> is unknown.

Although the effector macromolecule of p21<sup>ras</sup> is unknown, this protein's effector binding region has been localized to the region we have designated as G-2 (Fig. 2a, Table 1). Mutational replacements of residues in this region block neoplastic transformation<sup>56</sup> and also prevent interaction with GAP; the latter results form part of the evidence in favour of the proposal that GAP itself is an effector of p21<sup>ras</sup> (for summary and discussion, see refs 8, 57–60). In view of the fact that GTP alters the conformations of both regions G-2 and G-3, the latter region also should be considered a strong candidate for interaction with effectors. Certain mutations in G-3 do prevent GAP from stimulating GTPase activity<sup>61</sup>.

In the case of p21<sup>ras</sup>, little evidence supports a role for C-terminal sequences in activating effector function. Thus chimaeric genes encoding only the first 60 amino acids of v-Ha-ras and the remaining 129 residues of Rap1 are fully transforming<sup>62</sup>. Similarly, *ras* genes derived from fruitflies<sup>63</sup> or *S. cerevisiae*<sup>59</sup> can promote neoplastic transformation of mammalian cells; N-terminal sequences of these proteins resemble that of p21<sup>ras</sup>, whereas C-terminal sequences vary substantially<sup>35</sup>. On the other hand, chimaeras between v-Ha-ras and R-ras do not promote neoplastic transformation when the C-terminal 80 amino acids of the chimaera are derived from R-ras<sup>64</sup>, suggesting

TABLE 6 GAP interacts with G-2 region of Ras proteins

| GTPase             | Experimental observation                                                                                                                                                                                                                                                                                                                                                   | Interpretation                                                             |
|--------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| p21 <sup>ras</sup> | Point mutations that replace amino acids in G-2 prevent p21 <sup>ras</sup> interaction with GAP <sup>57,142,144,145</sup><br>Antibodies directed against G-2 inhibit p21 <sup>ras</sup> interaction with GAP <sup>148</sup><br>Synthetic peptides based on p21 <sup>ras</sup> sequence just proximal to G-2 inhibit p21 <sup>ras</sup> interaction with GAP <sup>149</sup> | G-2 interacts with GAP<br>G-2 interacts with GAP<br>G-2 interacts with GAP |
| R-ras              | G-2 sequence of R-ras is identical to that of p21 <sup>ras</sup> , and GAP stimulates GTPase of R-ras <sup>150</sup>                                                                                                                                                                                                                                                       | GAP of p21 <sup>ras</sup> recognizes conserved G-2 sequence                |
| Rap-1A             | G-2 sequence of Rap-1A is identical to that of p21 <sup>ras</sup> , and GAP of p21 <sup>ras</sup> binds tightly to Rap-1A but does not stimulate its GTPase <sup>147</sup>                                                                                                                                                                                                 | GAP of p21 <sup>ras</sup> recognizes conserved G-2 sequence                |
| Rho                | G-2 sequence differs from that of p21 <sup>ras</sup> and GAP of p21 <sup>ras</sup> does not stimulate rho's GTPase, but a different GAP does so <sup>150</sup>                                                                                                                                                                                                             | GAP of p21 <sup>ras</sup> does not recognize different G-2 sequence        |

that some specific C-terminal sequences may be required for effector activation.

# Conclusions

To perform their myriad cellular functions<sup>8</sup>, all GTPases go through the same unidirectional cycle, in which exchange of GDP for GTP turns on the switch and GTP hydrolysis turns it off. In each well studied case—for example EF-Tu in ribosomal protein synthesis, G protein  $\alpha$  chains in transmembrane signalling, and p21<sup>ras</sup> in control of cell proliferation—a GTP-induced change in its affinity for binding other macromolecules mediates the key cellular function of the GTPase. Structures of these GTPases resemble one another not only in overall design, but also in detail. Crystal structures of two examples, plus biochemical and genetic evidence from others, identify shared structural features involved in guanine nucleotide exchange, and common molecular mechanisms responsible for GTP hydrolysis and GTP-induced conformational change.

The close family resemblances already demonstrated show the potential utility of comparison and analogy in generating hypotheses regarding functions of GTPases. Investigations of one member of the family often offer hints for unravelling secrets of others. Thus, the roles of specific residues of p21<sup>ras</sup> in mediating guanine nucleotide exchange, GTP hydrolysis, or GTP-induced conformational change prompt experiments focused on cognate residues in other GTPases. Other examples—more adventurous but still amenable to experiment—are the proposals that G protein  $\alpha$  chains contain a built-in GAP element, that this element triggers GTP hydrolysis at a preset rate, and that it transmits GTP-induced conformational change from regions near the  $\gamma$ -phosphate of GTP to more distant regions of the  $\alpha$  chain.

These and other hypotheses, even more far-reaching, will soon be tested. We are just beginning to uncover the family's secrets. □

Henry R. Bourne is at the Departments of Pharmacology and Medicine and the Cardiovascular Research Institute, University of California, San Francisco, California 94143-0450, USA. Frank McCormick is at the Department of Molecular Biology, Cetus Corporation, Emeryville, California 94608, USA. David A. Sanders was formerly at the University of California and the Cetus Corporation and is now at the Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, USA.

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## ARTICLES

# Isotopic composition of atmospheric CO<sub>2</sub> inferred from carbon in C4 plant cellulose

Bruno D. Marino & Michael B. McElroy

Department of Earth and Planetary Sciences and Division of Applied Sciences, Harvard University, Cambridge, Massachusetts 02138, USA

The isotopic composition of atmospheric carbon dioxide provides an important constraint for models of the global carbon cycle. It is shown that carbon in C4 plants preserves an isotopic record of the CO<sub>2</sub> used in photosynthesis. Data for the maize plant *Zea mays* yield results for the isotopic composition of atmospheric CO<sub>2</sub> consistent with measurements of modern air and air trapped in polar ice. Data from C4 plants may thus be used to extend the isotopic record of atmospheric CO<sub>2</sub> into the past, complementing data from other sources.

IN principle, measurements of the isotopic composition of atmospheric CO<sub>2</sub>, specifically the ratio of <sup>13</sup>C to <sup>12</sup>C, can be used to distinguish the relative importance of oceanic and terrestrial biospheric exchange as mechanisms for removal of CO<sub>2</sub> from the atmosphere. Differences in the carbon isotopic composition of substances are conventionally expressed as δ<sup>13</sup>C values defining the parts per thousand (‰) deviation of the <sup>13</sup>C/<sup>12</sup>C ratio of a sample relative to that of the conventional carbonate standard PDB.

$$\delta^{13}\text{C} = \left\{ \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right\} \times 10^3 \text{‰} \quad (1)$$

Transfer of CO<sub>2</sub> from the atmosphere to the ocean results in a relatively small change in δ<sup>13</sup>C for atmospheric CO<sub>2</sub> (δ<sub>a</sub><sup>13</sup>) namely, an increase of 0.0027‰ per 10<sup>9</sup> t carbon (Gt C)<sup>1</sup>. In contrast, introduction of 1 Gt C to the atmosphere by transfer from the biosphere or by combustion of fossil fuel leaves a measurable isotopic imprint on atmospheric CO<sub>2</sub>—a decrease in δ<sub>a</sub><sup>13</sup> of ~0.026‰ (ref. 2). The value of δ<sub>a</sub><sup>13</sup> declined by ~0.55‰ between 1956 and 1978, from -6.69‰ to -7.24‰, whereas the concentration of CO<sub>2</sub> rose from 314 p.p.m. to 334 p.p.m. (ref. 3). The contemporary (1989) value of δ<sub>a</sub><sup>13</sup> is slightly less than -7.8‰, while the concentration of CO<sub>2</sub> is about 350 p.p.m.<sup>4</sup>. Although the rise in CO<sub>2</sub> for the pre-industrial and industrial period is well documented from a variety of measurements<sup>5–7</sup>, it has

proved remarkably difficult to construct a convincing budget for atmospheric CO<sub>2</sub>. The difficulty relates mainly to uncertainties in the rate at which carbon is exchanged with the ocean and terrestrial biosphere.

Direct measurements of δ<sub>a</sub><sup>13</sup> are available for a few clean-air locations on an almost continuous basis since 1978<sup>4</sup>. Except for a few measurements in 1955 and 1956<sup>8,9</sup>, earlier direct data are lacking. Atmospheric concentrations of CO<sub>2</sub> and values of δ<sub>a</sub><sup>13</sup>C have been obtained from studies of air trapped in polar ice<sup>10–13</sup>. There have been attempts to infer values of δ<sub>a</sub><sup>13</sup> from analysis of cellulose in tree rings<sup>14–16</sup>, but results in this case are ambiguous. Interpretation is difficult because large variations of δ<sup>13</sup>C can be observed around the circumference of a single ring in a single tree (up to 4‰<sup>15,17–21</sup>). These variations are thought to relate to factors attributable to physiological performance<sup>22,23</sup>.

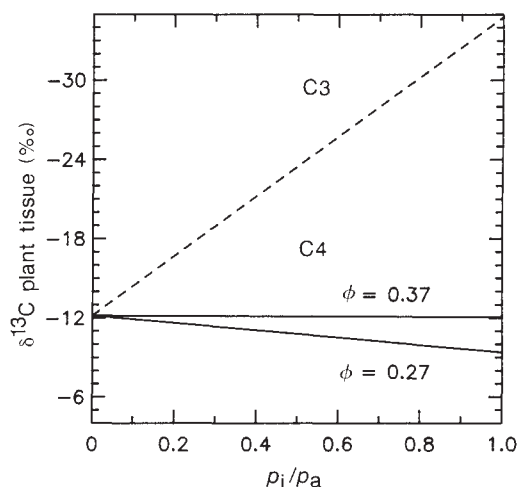


FIG. 1 Carbon isotope ratios of plant tissue (δ<sub>p</sub><sup>13</sup>) as a function of  $p_i/p_a$  (where  $p_i$  denotes the internal partial pressure of CO<sub>2</sub>,  $p_a$  the corresponding value for the partial pressure of CO<sub>2</sub> in the atmosphere) for C3 (dashed line) and C4 plants (solid lines). The relationships for C3 and C4 plants are given by equations (2) and (3) respectively. For C4 plants the value of δ<sub>p</sub><sup>13</sup> is independent of  $p_i/p_a$  when the bundle-sheath leakage factor  $\phi = 0.37$ . The dependence of δ<sub>p</sub><sup>13</sup> on  $p_i/p_a$  is indicated for *Zea mays* by the curve with  $\phi = 0.27$ .

TABLE 1 Identity, source and mean  $\delta^{13}\text{C}$  values of *Zea mays*

| Accession                              | Race or Cross         | Source                                                                                                                                     | Tissue | Mean $\delta^{13}\text{C}$ value | SD   | Number of samples ( <i>n</i> ) |
|----------------------------------------|-----------------------|--------------------------------------------------------------------------------------------------------------------------------------------|--------|----------------------------------|------|--------------------------------|
| <b>Clayton, North Carolina (1984)*</b> |                       |                                                                                                                                            |        |                                  |      |                                |
| 2533                                   | PCMG 83-1-10 × B73    | [[[(diploperennis teosinte × Palomero toluqueno) × elite inbred] × genetic tester stock] × genetic tester stock] × B73 (elite inbred line) | cob    | -10.33                           | —    | 1                              |
| 2537                                   | Funks G-5820          | commercial hybrid, partially exotic                                                                                                        | cob    | -10.45                           | 0.15 | 5                              |
| 2530                                   | AMB × 2024-21         | (isozyme genetic tester stock × (25% Balsas teosinte, 25% Huevito from Venezuela, 50% elite US inbreds))                                   | cob    | -10.67                           | 0.29 | 14                             |
|                                        |                       |                                                                                                                                            | kernel | -11.03                           | 0.06 | 5                              |
|                                        |                       |                                                                                                                                            | husk   | -11.30                           | 0.26 | 5                              |
|                                        |                       |                                                                                                                                            | stem   | -11.27                           | 0.20 | 5                              |
| 2456                                   | Cubano Tuson          | ECU 653, Ecuador                                                                                                                           |        | -10.64                           | 0.19 | 5                              |
| 2455                                   | Cuban Amarillo Duro   | ECU 542, Ecuador                                                                                                                           |        | -11.4                            | 0.14 | 2                              |
| 2461                                   | Cristaleno Norteno    | CHI 338, Chile                                                                                                                             |        | -10.23                           |      | 1                              |
| 2468                                   | Dente R. G. Rugoso    | RGS 587, Brazil                                                                                                                            |        | -10.90                           | 0.19 | 2                              |
| 2473                                   | Pororo                | Bov 587, Bolivia                                                                                                                           |        | -11.03                           | 0.25 | 2                              |
| 2474                                   | Pororo                | Bov 806, Bolivia                                                                                                                           |        | -11.29                           | 0.25 | 4                              |
|                                        |                       |                                                                                                                                            |        | -10.75                           | 0.36 | 36                             |
| <b>Homestead, Florida (1987)†</b>      |                       |                                                                                                                                            |        |                                  |      |                                |
| 6501                                   | Nal-Tel               | YUC 7 CIMMYT, Mexico                                                                                                                       |        | -10.41                           | 0.10 | 5                              |
| 6500                                   | Chapalote             | SIN 2 CIMMYT, Mexico                                                                                                                       |        | -10.40                           | 0.20 | 5                              |
| 6502                                   | Maize-teosinte hybrid | W629 (standard line) D079-12 (Central Plateau)                                                                                             |        | -10.08                           | 0.14 | 2                              |
| 6503                                   | Maize-teosinte hybrid | MGKN (genetic tester stock) × D079-19 (Balsas)                                                                                             |        | -10.32                           | 0.06 | 2                              |
| 6506                                   | Maize-teosinte hybrid | 1290-19 (genetic tester stock) × D027-19 (Jalisco)                                                                                         |        | -10.81                           | 0.37 | 2                              |
|                                        |                       |                                                                                                                                            |        | -10.42                           | 0.26 | 15                             |

\* All accessions were grown in adjacent plots at the Central Crops Research Station, Clayton, North Carolina. Watering was by natural rainfall and pond irrigation. The plants were hand-pollinated by sibbing.

† All accessions were grown in adjacent plots by the Agricultural Alumni Seed Improvement Association, Inc. at Homestead, Florida. Watering was by natural rainfall and groundwater irrigation. The plants were hand-pollinated by sibbing.

It is clear that a reliable plant proxy for  $\delta_a^{13}$  would be valuable. Plants, in principle, can provide information on the spatial variability of  $\delta_a^{13}$ . They are capable of much finer time resolution (seasonal in some cases) than ice cores, which must of necessity integrate over relatively long time intervals<sup>24</sup>, and do not require corrections for  $\text{N}_2\text{O}^{25}$  or gravitational fractionation<sup>26</sup>. We argue that, although problems associated with interpretation of the isotopic composition of carbon in tree rings may be insuperable, comparable information may be obtained from studies of carbon in the cellulose of C4 plant material. We present results for  $\delta^{13}\text{C}$  in cellulose nitrate (prepared as in ref. 27) of modern races of the maize plant *Zea mays* grown in a variety of temporal and spatial contexts. Comparisons with directly measured and ice-core values of  $\delta_a^{13}$  indicate excellent agreement, demonstrating that carbon in maize, and possibly other C4 plants, can be used as a proxy for the isotopic composition of atmospheric  $\text{CO}_2$ .

### Physiological influences

The mechanism for fixation of carbon by C3 plants is fundamentally simpler than for C4 plants. Carbon dioxide enters the leaf through the stomata in both cases. It diffuses from the mesophyll in C3 plants, where it is fixed by the primary carboxylating enzyme, ribulose-1,5-bisphosphate carboxylase ( $\text{RuP}_2$ ) (ref. 28). Diffusion results in a small enrichment of  $^{12}\text{C}$  relative to  $^{13}\text{C}$ ; the associated change in  $\delta^{13}\text{C}$  is 4.4‰<sup>29</sup> (following Farquhar *et al.*<sup>30</sup>, we use positive numbers to indicate changes resulting in a lower value of  $\delta^{13}\text{C}$ ). Further enrichment of  $^{12}\text{C}$  occurs through the action of  $\text{RuP}_2$ ; the change in  $\delta^{13}\text{C}$  in this case is 27‰<sup>31,32</sup>.

Farquhar *et al.*<sup>30</sup> developed a simple expression relating the value of  $\delta^{13}\text{C}$  for carbon fixed by a C3 plant to  $\delta^{13}\text{C}$  of the external environment:

$$\delta_p^{13} = \delta_a^{13} - a - (b - a)p_i/p_a \quad (2)$$

where  $\delta_a^{13}$  defines the isotopic value of atmospheric  $\text{CO}_2$  used by the plant during photosynthesis,  $\delta_p^{13}$  denotes the isotopic value of the photosynthate produced in the leaf from which

plant tissue is made,  $a$  is the change in  $\delta^{13}\text{C}$  due to diffusion (4.4‰),  $b$  is the change introduced by the action of  $\text{RuP}_2$  (27‰) and  $p_i$  and  $p_a$  denote the partial pressures of  $\text{CO}_2$  in the atmosphere and in the intercellular leaf space. Equation (2) allows two simple limiting cases. When fixation is limited primarily by the supply of  $\text{CO}_2$  through the stomata, due, for example, to large vapour pressure deficits,  $p_i/p_a$  is small and the difference between  $\delta^{13}\text{C}$  of the plant and the atmosphere approaches the value determined by diffusion,  $a$ . In the other extreme, when fixation is limited mainly by the action of  $\text{RuP}_2$ , due, for example, to very low light intensities or nutritional deficiencies,  $p_i/p_a$  approaches 1 and the difference between  $\delta^{13}\text{C}$  of the plant and atmosphere is determined largely by fractionation associated with  $\text{RuP}_2$ . The variation of  $\delta_p^{13}$  as a function of  $p_i/p_a$  is illustrated for C3 plants in Fig. 1.

Carbon dioxide dissolves in the cell sap on entering C4 plants and is converted to  $\text{HCO}_3^-$  through the action of carbonic anhydrase. It is transformed subsequently to oxalacetate by phosphoenolpyruvate carboxylase (PEP). As with C3 plants, diffusion results in enrichment of  $^{12}\text{C}$  relative to  $^{13}\text{C}$  (4.4‰). Conversion to  $\text{HCO}_3^-$  enriches  $^{13}\text{C}$  relative to  $^{12}\text{C}$  (the change in  $\delta^{13}\text{C}$  at 25 °C is equal to -7.9‰, assuming equilibrium between  $\text{CO}_2$  and  $\text{HCO}_3^-$  (ref. 33)). PEP favours  $^{12}\text{C}$ , with an associated change in  $\delta^{13}\text{C}$  of 2.2‰, resulting in a net change in  $\delta^{13}\text{C}$  with respect to the atmosphere of -1.3‰ ( $4.4 - 7.9 + 2.2$ ). Following various transformations,  $\text{CO}_2$  is released in the bundle-sheath cells and is fixed by  $\text{RuP}_2$ . A portion of the  $\text{CO}_2$  in the bundle-sheath cells leaks back to the mesophyll to be recycled by PEP. Leakage and recycling causes the final isotopic composition of plant carbon to depend on the fractionation associated with  $\text{RuP}_2$ , as with C3 plants. Farquhar<sup>34</sup> presents a simple expression relating  $\delta_p^{13}$  for C4 plants to  $\delta_a^{13}$ :

$$\delta_p^{13} = \delta_a^{13} - a - (c + b\phi - a)p_i/p_a \quad (3)$$

The isotopic shifts  $a$  and  $b$  are the same as the quantities introduced for C3 plants;  $c$  is the isotopic shift associated with the combined influences of carbonic anhydrase and PEP (-7.9 +



2.2 = -5.7%), whereas  $\phi$  is the fraction of  $\text{CO}_2$  released in the bundle-sheath cells returned to the mesophyll. Values of  $\delta_p^{13}$  for selected values of  $\phi$  are shown as functions of  $p_i/p_a$  for C4 plants in Fig. 1.

The fractionation between C4 plants and the environment varies linearly as a function of  $p_i/p_a$  for fixed values of  $\phi$ , with a slope given by  $(27\phi - 10.1)\%$ , assuming the values for  $a$ ,  $b$  and  $c$  given above. The variation of  $\delta_p^{13}$  with  $p_i/p_a$  is much less for C4 than for C3 plants. Indeed, in the limiting case when  $\phi = 0.37$ ,  $\delta_p^{13}$  is independent of  $p_i/p_a$ . Hattersley<sup>35</sup> and Bowman *et al.*<sup>36</sup> measured values of  $\phi$  for *Zea mays* leaves of 0.26 and 0.27 respectively. We adopt a value for *Zea mays* of  $\phi = 0.27$ . *Zea mays*, a monocot grass, belongs to the NADP-ME (nicotinamide adenine dinucleotide malic enzyme) subgroup of C4 plants; the conductance associated with leakage from the bundle sheaths is low because a suberized lamella is present in the bundle sheath<sup>37</sup>. This may account for the relatively small value of  $\phi$ . Wong *et al.*<sup>38</sup> found that *Zea mays* maintains a nearly constant value of 0.4 for  $p_i/p_a$ , even under conditions where the capacity of the mesophyll to fix carbon is altered by subjecting the plants to varying supplies and quality of nutrition<sup>39</sup>, to a range of light levels<sup>40</sup> and to a variable supply of water<sup>41</sup>. Here we adopt the value 0.4 for  $p_i/p_a$ , but note that results for  $\delta_a^{13}$  should be relatively insensitive to uncertainties in this parameter (see curve for *Zea mays* in Fig. 1). With our choice of values for  $p_i/p_a$  and  $\phi$ , the relation between  $\delta_p^{13}$  and  $\delta_a^{13}$  for *Zea mays* is given by:

$$\delta_a^{13} = 3.276 + \delta_p^{13} \quad (4)$$

It should be stressed that the relation defined by equation (4) is approximate. For a discussion of the complexities associated with isotopic transformations of carbon under less idealized conditions in plants, see ref. 42. For example, the integrated signature of plants with either photosynthetic pathway may be influenced by dissimilatory processes such as dark respiration and photorespiration<sup>43</sup>. Isotopic shifts due to photorespiration can be largely ignored in C4 plants, because they have little or no light-dependent oxygenase activity *in vivo* for atmospheric concentrations of  $\text{O}_2$ <sup>44</sup>.

## Results for *Zea mays*

We used a variety of racial accessions (a restricted catalogue number of a specific maize type) and experimental crosses of *Zea mays* grown in Clayton, North Carolina, in 1984, in combination with similar plants for 1987 from Homestead, Florida. Plants in both locations were grown side by side in the same fields under the same conditions. Measurements of  $\delta^{13}\text{C}$  were obtained from analysis of the  $\text{CO}_2$  derived from combustion of cellulose nitrate: 14 replicate analyses of cob and kernel cellulose nitrate yielded a mean  $\Delta\delta$  of 0.06 with a standard deviation (SD) of 0.07‰. An internal standard (pure alpha cellulose) included in 21 separate sets of nitrations indicated a mean and SD of  $-23.87 \pm 0.07\%$ . From these data, we estimate the overall external precision to be better than 0.10‰. Cobs, kernels, husk and stem tissue were analysed separately in the case of samples from Clayton. The data for Homestead are for cobs only. The analysis includes 36 plants, 9 different accessions, for Clayton; 15 plants, 5 accessions, from Homestead.

Cobs were selected at random, one from each plant. The average value of  $\delta^{13}\text{C}$  for cellulose nitrate in cobs from Clayton, was  $-10.75 \pm 0.36\%$  (36 samples). The corresponding average for cobs from Homestead was  $-10.42 \pm 0.26\%$  (15 samples). Values of  $\delta^{13}\text{C}$  for cellulose nitrate exhibit modest variability ( $<0.5\%$ ) with position in individual cobs—significantly smaller than the variability (up to 4‰) reported<sup>21,45</sup> for cellulose in individual tree rings. Analyses of distinct fragments of a cob from a plant of accession 2530 (rachis, pith and floral bract) gave values for  $\delta^{13}\text{C}$ , which agreed with the mean value for the entire cob to within  $\pm 0.25\%$ .

Values of  $\delta^{13}\text{C}$  were also determined from cellulose nitrate

extracted from kernels of 5 plants from accession 2530 grown in Clayton. Kernels of individual plants were combined; the average value of  $\delta^{13}\text{C}$  was  $-11.03 \pm 0.06\%$ . Using this value for  $\delta_p^{13}$  in equation (4), we estimate a value for  $\delta_a^{13}$  of  $-7.75\%$ , in excellent agreement with the globally averaged, seasonally adjusted value of  $-7.71\%$  reported<sup>4</sup> for the summer of 1984. Husks and stems of the five plants were analysed and the values of  $\delta^{13}\text{C}$  in this case were  $-11.30 \pm 0.26\%$  and  $-11.27 \pm 0.20\%$  respectively (five samples in each case). Table 1 shows the results for all analyses. The values of  $\delta_a^{13}$  are derived from kernels using equation (4); for cobs we used the modified relation

$$\delta_a^{13} = 2.916 + \delta_{\text{cob}}^{13} \quad (5)$$

suggested by the difference in the means of  $\delta^{13}\text{C}$  values for kernels and cobs obtained for accession 2530 from Clayton. We estimate a mean value for  $\delta_a^{13}$  of  $-7.83\%$  for cobs, which also agrees well with the globally averaged value for the summer of 1984,  $-7.71\%$  (ref. 4). Using the mean value of  $\delta^{13}\text{C}$  for cobs grown during the winter of 1987 in Homestead, we obtain a value for  $\delta_a^{13}$  of  $-7.50\%$ , in agreement with the globally averaged value of  $-7.67\%$  (ref. 4).

## Secular trends in $\delta_a^{13}$

Results from the analysis of cellulose nitrate extracted from the kernels of maize grown in the same fields in Ames, Iowa, from 1948 to 1986 are shown in Fig. 2. Each data point represents a combined analysis of  $\sim 100$  kernels taken from a bulk sample of 100 ears of a specific type from 100 different plants. Eight random replicate analyses covering the years 1948, 1950, 1952,

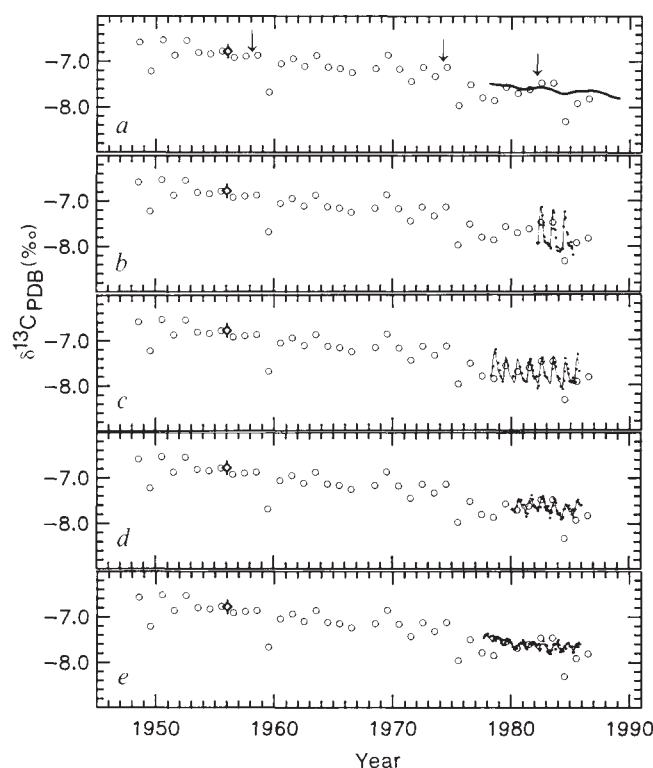


FIG. 2 Values of  $\delta_a^{13}$  as a function of time. Open circles represent values of  $\delta_a^{13}$  inferred from measurements of  $\delta^{13}\text{C}$  in maize kernel cellulose nitrate using equation (4). Samples were grown in Ames, Iowa ( $42^\circ\text{N}$ ,  $93^\circ\text{W}$ ); each point represents a composite of kernels for a single year. Small dots and solid lines represent directly measured values of  $\delta_a^{13}$  and spline fits, respectively, from Keeling *et al.*<sup>4</sup> as follows. *a*, Monthly averaged, seasonally adjusted mean of  $\delta_a^{13}$  for Mauna Loa and the South Pole; *b-e*, monthly averaged records for Point Barrow, Alaska ( $71^\circ\text{N}$ ), La Jolla, California ( $33^\circ\text{N}$ ), Mauna Loa, Hawaii ( $20^\circ\text{N}$ ) and Fanning and Christmas Islands ( $2-4^\circ\text{N}$ ), respectively. The solid diamond indicates measurements for 1955-56 (ref. 9) corrected by  $+0.23\%$  for  $\text{N}_2\text{O}$  interference (ref. 2). Arrows in (*a*) indicate years of 'strong' or 'very strong' El Niño events as defined in ref. 48.

1955, 1961, 1972, 1973 and 1978 yielded a mean difference in  $\delta^{13}\text{C}$  of  $0.09 \pm 0.06\%$ . The type of maize varied through the sequence; different types grown in each of four years (1948, 1957, 1968, 1982) had differences in  $\delta^{13}\text{C}$  of  $0.09 \pm 0.09\%$ . We estimate the overall precision of the data to be better than 0.10%. The data show remarkable consistency; a clear secular trend towards lighter  $\delta^{13}\text{C}$  values is discernible.

The Ames sequence should provide a growth-weighted estimate of the seasonally averaged value of  $\delta_a^{13}$  (growth period is typically from May to September). The concentration of atmospheric  $\text{CO}_2$  decreases rapidly during May from its winter maximum to the summer minimum, with an associated change of  $\delta_a^{13}$  in the opposite sense, from depletion to enrichment of  $^{13}\text{C}$  (ref. 4). The seasonal amplitude of the change in  $\delta_a^{13}$  measured at Mauna Loa Observatory in Hawaii ( $19.5^\circ\text{N}$ ,  $155.6^\circ\text{W}$ ) is  $\sim 0.3\%$ , increasing to  $\sim 0.8\%$  further north at Point Barrow, Alaska ( $71.3^\circ\text{N}$ ,  $156.6^\circ\text{W}$ )<sup>4</sup>. We expect the seasonal range at Ames to lie between these extremes. The relatively light values of  $\delta_a^{13}$  inferred for 1949, 1959, 1976 and 1984 could reflect differences in the weighting of  $\delta_a^{13}$  by the plants, due to shifts in the seasonal change in  $\delta_a^{13}$  with respect to the growth sequence of the plants.

Figure 2 includes a comparison of the kernel-derived values of  $\delta_a^{13}$  with direct measurements for atmospheric  $\text{CO}_2$  (ref. 4). The maize data indicate a long-term decline in  $\delta_a^{13}$  of  $-0.031\% \text{ yr}^{-1}$ , in excellent agreement with the decrease (of  $-0.031\% \text{ yr}^{-1}$ ) implied by the modern measurements, obtained by combining the 1956 measurement with the annual average of the 1988 seasonally adjusted data for Mauna Loa and the South Pole (Fig. 2a), and  $-0.028\% \text{ yr}^{-1}$  defined using the complete record from Mauna Loa and the South Pole for the period 1978 to 1988. The estimated confidence interval for the slope of the Ames data is  $-0.031 \pm 0.006$ , assuming a critical value of 0.05.

Figure 3a compares the kernel-derived values of  $\delta_a^{13}$  with direct measurements by Keeling *et al.*<sup>4</sup>, and with measurements by Friedli *et al.*<sup>10</sup> for gas trapped in ice at Siple Station, Antarctica. The ice data cover the period 1744–1953. The uncertainty in age associated with air in a particular bubble (defined as the time required for the bubble volume to grow from 10 to 90% of

its final size) is estimated at 22 years. The Siple data and the Ames data intersect, indicating very good agreement between the two data sets. Figure 3b presents a plot of  $\delta_a^{13}$  as a function of  $\text{CO}_2$  concentration. Concentrations of  $\text{CO}_2$  before 1958 were taken from the ice-core measurements<sup>7,10</sup>; data for subsequent years are from ref. 4. The value of  $\delta^{13}\text{C}$  for fossil-fuel-derived  $\text{CO}_2$  is  $\sim -27.28\%$  (ref. 46). If all the fossil-fuel  $\text{CO}_2$  released were to remain in the atmosphere, we would expect a change in  $\delta_a^{13}$  of  $\sim -0.025\% \text{ p.p.m.}^{-1}$  (ref. 2). The change in  $\delta_a^{13}$ , measured with respect to the  $\text{CO}_2$  concentration taken from the Siple data, is about  $-0.011\% \text{ p.p.m.}^{-1}$  before 1956, increasing to  $-0.028\% \text{ p.p.m.}^{-1}$  in the more recent period, 1956–86, as indicated by the Ames data. This is in excellent agreement with the rate of change of  $-0.028\% \text{ p.p.m.}^{-1}$  deduced from the measurements of Keeling *et al.*<sup>4</sup> for the same period. The time constant for the atmosphere to equilibrate isotopically with the ocean is appreciably less than that associated with equilibration of the partial pressure of  $\text{CO}_2$  (ref. 5). The increase in the slope of  $\delta_a^{13}$  with  $\text{CO}_2$  evident for the later years in Fig. 3a most probably reflects departures from isotopic equilibrium associated with rapid acceleration in recent years of the release of  $\text{CO}_2$  from the combustion of fossil fuel.

The agreement between the maize data and the combination of ice-core and modern air measurements, both with respect to long-term trends and absolute values of  $\delta_a^{13}$ , strongly supports the use of cellulose in corn as a proxy for the isotopic composition of  $\text{CO}_2$  in the atmosphere. The maize data provide a bridge between the ice-core measurements and the extensive record of direct measurements available since 1978.

### Spatial and temporal records

Results for  $\delta_a^{13}$ , obtained using equation (5), with measurements of  $\delta^{13}\text{C}$  in cellulose nitrate extracted from cobs grown at 21 geographically distributed stations, in 1987, are shown in Fig. 4, which includes a comparison with measurements for 1987 at Point Barrow (Alaska), La Jolla (California), Hawaii and the Line Islands (C. D. Keeling, personal communication). The value for  $\delta_a^{13}$  of  $-7.60 \pm 0.26\%$ , inferred from cobs from 5 remote locations (Hawaii, Ethiopia, Finland, Alaska and Mexico),

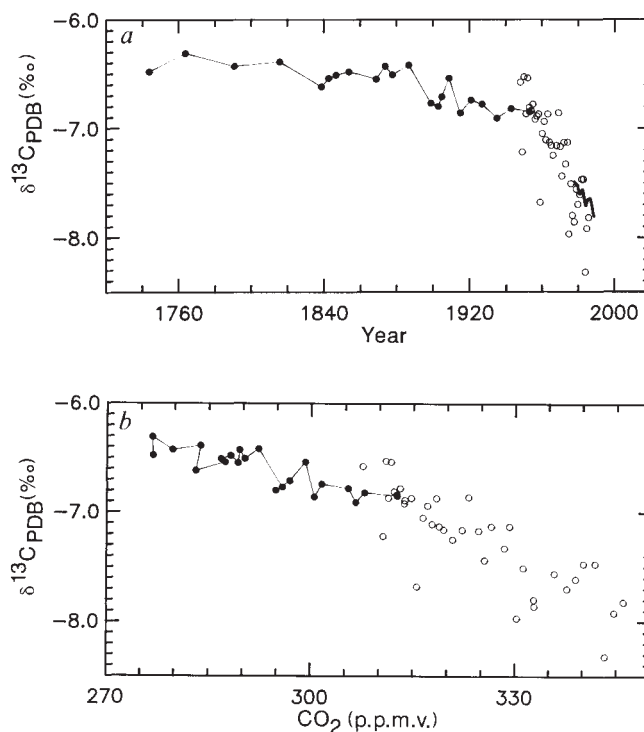
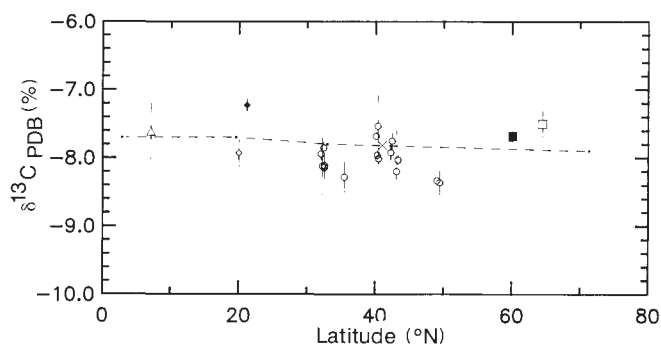


FIG. 3 a, Values of  $\delta_a^{13}$  for  $\text{CO}_2$  (closed circles) from the Siple Station ice core ( $75^\circ\text{N}$ ) compared with kernel derived values of  $\delta_a^{13}$  from Ames, Iowa (open circles) as a function of time. The solid line represents  $\delta_a^{13}$  values measured by Keeling *et al.*, 1989). b, Values of  $\delta_a^{13}$  as a function of the atmospheric concentration of  $\text{CO}_2$ . Siple Station values of  $\delta_a^{13}$  (solid circles) are plotted against  $\text{CO}_2$  concentrations measured in corresponding ice samples<sup>10</sup>. Kernel-derived values of  $\delta_a^{13}$  from Ames, Iowa are plotted against  $\text{CO}_2$  concentrations measured at Mauna Loa and the South Pole<sup>4</sup>.

FIG. 4 Values of  $\delta_a^{13}$  inferred from maize cobs as a function of the latitude of growth using equation (4). All cobs were grown during the 1987 growing season. Remote locations are: Addis Ababa, Ethiopia (open triangle); El Batan, Mexico (open diamond); Waimanalo, Hawaii (solid diamond); Helsinki, Finland (solid square); Fairbanks, Alaska (open square). Open circles represent results for a variety of locations within the continental United States. The large X represents the value of  $\delta_a^{13}$  inferred from kernels grown in Ames, Iowa in 1986. Dots and dashed line represent  $\delta_a^{13}$  values measured for 1987 (ref. 4): (1), Point Barrow, Alaska (71° N); (2), La Jolla, California (33° N); (3), Mauna Loa, Hawaii (20° N); and (4), Fanning and Christmas Islands (2–4° N).



agrees well with the globally averaged value of  $-7.68 \pm 0.02\%$  for 1987 (ref. 4). Data for  $\delta_a^{13}$  obtained from cobs grown in the continental United States (16 locations) have a relatively large scatter,  $\pm 0.23$  around a mean of  $-8.02\%$ . An average of the full set of cob data in Fig. 4 indicates a value of  $-7.92\%$  with an uncertainty of  $\pm 0.29\%$ , in reasonable agreement with the globally averaged result,  $-7.68\%$ . A portion of the variance in the continental data shown in Fig. 4 could reflect the influence of isotopically light  $\text{CO}_2$  associated with local or regional sources of  $\text{CO}_2$  from combustion of fossil fuel. An additional contribution could be associated with specific genetic, physiological or environmental effects on the fractionation of carbon in maize plants. The data presented earlier for corn from Clayton, North Carolina, indicated fractionation between kernels and cobs of  $0.36\%$ . Cobs from a variety of genetically diverse plants showed a variance in  $\delta^{13}\text{C}$  of  $\pm 0.36\%$ . Cobs contain several distinct types of tissue, rachis, pith and floral bract, which show small variations relative to the whole cob ( $\pm 0.25\%$ ); different races can have varying amounts of these tissues. The scatter of results in Fig. 4 may simply indicate inherent uncertainty in recovery of  $\delta_a^{13}$  from measurements of  $\delta^{13}\text{C}$  in the cellulose nitrate of cobs.

Cobs from Clayton were harvested at three different times during the growing season in 1987; on 7 July, 13 August and 8 October, after 64, 102 and 158 days of growth respectively.

Values of  $\delta_a^{13}$  derived for these dates using equation (4) were  $-8.87 \pm 0.07$ ,  $-8.04 \pm 0.19$ , and  $-8.28 \pm 0.20\%$  respectively. These results are consistent with the change in  $\delta_a^{13}$  inferred from measurements of  $\text{CO}_2$  concentrations over the same period at Key Biscayne, Florida (K. W. Thoning, personal communication). Using the seasonal relationship between  $\text{CO}_2$  and  $\delta_a^{13}$  obtained from measurements at La Jolla,<sup>47</sup> we estimate July, August and October values for  $\delta_a^{13}$  of  $-8.56$ ,  $-8.38$  and  $-8.42\%$ , respectively, in agreement both in magnitude and temporal trend with the results inferred from cobs.

## Conclusions

The isotopic composition of carbon in the cellulose nitrate of C4 plants, *Zea mays* in particular, has been shown to provide a useful record of the isotopic composition of atmospheric  $\text{CO}_2$ . The data presented here emphasize the period since 1948. Samples of corn are available with high time resolution dating back to AD 1500; results of analyses of these materials will be described elsewhere. Samples of C4 material exist on a more limited basis back to the end of the last ice age. Studies of intact and fossil C4 material should provide valuable information on the status of the global carbon cycle through time and may be expected to highlight changes in the mobilization of carbon in the past, providing a useful complement to data from both deep-sea and polar ice cores. □

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# Cyclin is degraded by the ubiquitin pathway

Michael Glotzer, Andrew W. Murray\* & Marc W. Kirschner

Departments of Biochemistry and Biophysics and \* Physiology, School of Medicine, University of California at San Francisco, San Francisco, California 94143-0448, USA

Cyclin degradation is the key step governing exit from mitosis and progress into the next cell cycle. When a region in the N terminus of cyclin is fused to a foreign protein, it produces a hybrid protein susceptible to proteolysis at mitosis. During the course of degradation, both cyclin and the hybrid form conjugates with ubiquitin. The kinetic properties of the conjugates indicate that cyclin is degraded by ubiquitin-dependent proteolysis. Thus anaphase may be triggered by the recognition of cyclin by the ubiquitin-conjugating system.

PROTEOLYSIS plays a critical part in mitosis of the eukaryotic cell cycle. Mitosis is induced by the activation of M-phase promoting factor (MPF), a protein kinase whose principal subunits are p34<sup>cdc2</sup>, which has extensive homology to known protein kinases, and cyclin, a regulatory subunit whose abundance fluctuates throughout the cell cycle<sup>1</sup>. The transition from metaphase to anaphase which marks the end of mitosis is induced by the degradation of cyclin, which in turn leads to the inactivation of MPF. As cells enter interphase, cyclin degradation ceases, cyclin accumulates and, as a result of a complex series of post-translational reactions, MPF is activated<sup>2</sup> and another round of mitosis ensues. Cyclin degradation is thus the crucial event in exiting metaphase and entering the next interphase. As expected, cyclin mutants that retain the ability to activate MPF but cannot be degraded arrest the cell cycle in mitosis<sup>3</sup>. The basic features of this scheme appear to be conserved in all eukaryotic cells, although several other regulators, such as the products of the *Schizosaccharomyces pombe* genes *cdc25* and *wee1*, may also play a part in regulating the activity of MPF (ref. 4). The regulation of cyclin degradation is poorly understood, but it is clear that cyclin proteolysis is triggered by the presence of active MPF (ref. 5). Once initiated, cyclin degradation is rapid and highly specific.

Protein levels are determined by the balance between the rates of protein synthesis and protein degradation<sup>6</sup>. The half-lives of intracellular proteins vary over at least three orders of magnitude from minutes to days<sup>6</sup>, and a number of distinct pathways of intracellular proteolysis have been described<sup>7</sup>. One prominent degradation pathway is mediated by conjugation of ubiquitin to unstable proteins<sup>8-10</sup>. Ubiquitin is a highly conserved protein of relative molecular mass 7,000 (*M<sub>r</sub>* 7K) which can be covalently linked through isopeptide bonds to lysine residues on proteins; when proteins are multiubiquitinated at a single site (by isopeptide linkage to lysine 48 of a ubiquitin molecule that is already ligated to a protein degradation substrate) they become targets for degradation<sup>11</sup>. The proteins involved in ubiquitin conjugation have been extensively characterized by both biochemical and genetic analysis<sup>8-10,12-14</sup>. However, there is only one case where a specific protein of physiological interest has been shown to be degraded by the ubiquitin pathway<sup>15</sup>.

We have used crude extracts prepared from *Xenopus* eggs to examine several aspects of the degradation of cyclin. We have generated a stable metaphase state where cyclin is constitutively degraded. Using this system we have shown that the N terminus of cyclin is not only necessary but also sufficient for M-phase-specific degradation. Mutational analysis has localized the

region of the N terminus that is required for cell-cycle-regulated destruction of cyclin. Finally, we have shown that cyclin degradation at mitosis is accompanied by the formation of cyclin-ubiquitin conjugates. A mutation within cyclin that inhibits degradation also inhibits ubiquitin conjugation. The measured flux through the cyclin-ubiquitin intermediate suggests that cyclin is degraded by way of the ubiquitin pathway. These results suggest that MPF regulates the conjugation of ubiquitin to cyclin, leading to the rapid destruction of cyclin by the constitutively active, ubiquitin-dependent proteolytic system.

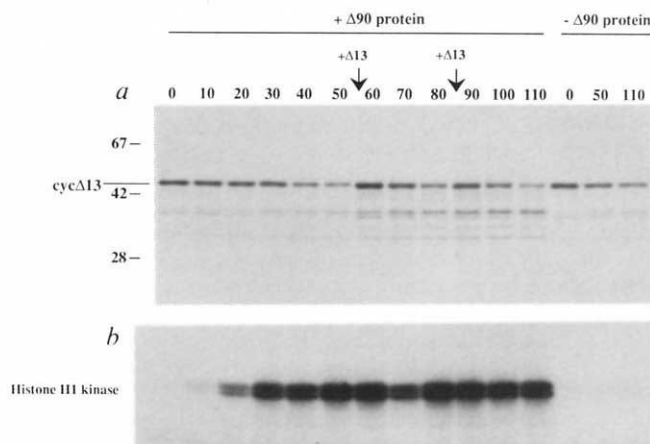
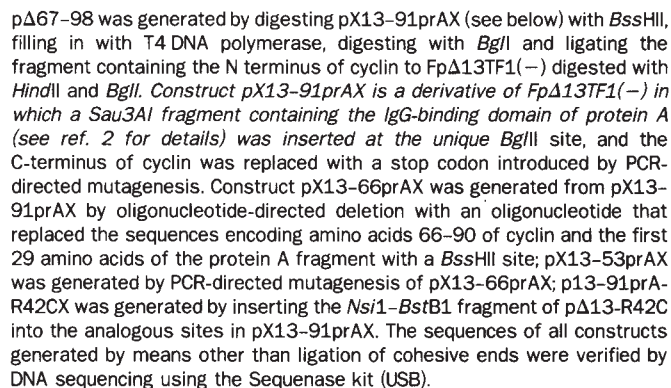


FIG. 1  $\Delta 90$  (stable mitotic) extracts constitutively degrade cyclin. Interphase extracts were prepared as described<sup>2</sup>. At 0 min, 1/10 volume of translation mix containing <sup>35</sup>S-labelled cycΔ13 was added, 1/20 volume of  $\Delta 90$  protein was added to a portion of the extract, and the extracts were incubated at 22 °C. More cycΔ13 was added to the extracts containing  $\Delta 90$  protein at 0, 59 and 89 min. At the indicated times samples were removed for analysis of <sup>35</sup>S-protein by SDS-PAGE and autoradiography, and samples were also analysed for histone H1 kinase activity as described<sup>2</sup>. It should be noted that cyclin destruction in mitotic extracts was biphasic; 80% of the cyclin was rapidly degraded but 20% was not. This is not seen *in vivo* and appears to be a consequence of a fraction of inactive cyclin molecules translated in the reticulocyte lysate.

**METHODS.** An expression vector for cycΔ90, pT7Δ90, was created by cutting cycΔ13 with *Bgl*II followed by partial digestion with *Bam*H1. The resulting 1.1-kb fragment was ligated into the T7 translation vector pET3b (ref. 38) linearized at the unique *Bam*H1 site. One litre of LB containing *E. coli* strain BL21(DE3)pLysS, pT7Δ90 was grown to 37 °C to OD<sub>600</sub> 0.7.  $\Delta 90$  protein was induced for 1 h with 0.08 mg ml<sup>-1</sup> IPTG. The cells were pelleted, washed with 0.9% NaCl, pelleted and resuspended in 25 ml buffer A (10 mM Tris, 50 mM NaCl, 1 mM EDTA, pH 8.0) also containing 5 mM DTT, 0.05% Nonidet P-40, and 10 μg ml<sup>-1</sup> chymostatin, pepstatin and leupeptin. The extract was sonicated for 2 min on ice and spun in a Sorvall SS-34 rotor at 12,000 r.p.m. for 15 min at 4 °C. The pellet was washed in buffer A containing 0.5 M NaCl (final), and resuspended in 20 ml buffer A containing 8 M urea and 5 mM DTT. Buffer B (20 ml; 50 mM Tris, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM DTT) was slowly added and the solution was spun 5 min 12,000 r.p.m. in a Sorvall HB-4 rotor at 4 °C. The supernatant was dialysed against buffer B. The resulting preparation contained ~0.5 mg ml<sup>-1</sup> cycΔ90 protein, which was about 80% pure. <sup>35</sup>S-labelled cycΔ13 was produced by translation in a reticulocyte lysate system (Promega) of RNA transcribed by T7 RNA polymerase from FpΔ13TF1(-), a derivative of cycΔ13 (ref. 3), pGEM2 (Promega), pSP64T (ref. 39), and pUC-f1 (Pharmacia). It consists of the coding sequence of cycΔ13 flanked by the 5' and 3' untranslated regions of β globin originally derived from pSP64T (the 5'-untranslated region of β globin had been mutated to create a consensus ribosome binding site for *E. coli* translation) and inserted into the polylinker of pGEM2 that had the f1-ori inserted at the unique *Sph*I site.



**METHODS.** Mitotic extracts were generated by addition of 1/20 volume of  $\Delta 90$  protein to fresh interphase extracts followed by incubation for 1 h at 22 °C. After 1 h sucrose was added to a final concentration of 150 mM and the extracts were frozen in small aliquots in liquid nitrogen. Frozen extracts were used in all experiments in this paper except those for Fig. 3. The  $^{35}\text{S}$ -labelled derivatives containing the C-terminal half of cyclin (constructions outlined below) were transcribed *in vitro* and translated in reticulocyte extracts, and 1/5 volume was added to extracts. The  $^{125}\text{I}$ -labelled derivatives, which are protein A fusions, were expressed in BL21(DE3)pLysS, purified by affinity chromatography with IgG-Sepharose 6 FF (Pharmacia) according to the manufacturer's instructions, and radiolabelled by iodination with 2 mM chloramine T (Eastman Kodak) with 0.5 mCi  $\text{Na}^{125}\text{I}$  (NEN) diluted to a specific activity of 10 mCi  $\text{ml}^{-1}$ . After 2 min the labelling reaction was stopped by addition of DTT to 0.1 M, carrier BSA was added to 0.2  $\text{mg ml}^{-1}$  and the unincorporated radioactivity was removed by a G-25 Sephadex spin column equilibrated with 10 mM potassium phosphate, 2 mM DTT, pH 7.8. The only tyrosine residues that could be modified by the iodination reaction are on the protein A moiety. The iodinated proteins, labelled to a specific activity of  $10^6$  c.p.m. per  $\mu\text{g}$ , were added to extracts at a final concentration of 5,000 c.p.m. per  $\mu\text{l}$ . The constructs were generated as follows. p $\Delta 13\text{R}42\text{C}$  was produced by oligonucleotide-directed mutagenesis<sup>49</sup> from Fp $\Delta 13\text{T}1\text{F}1(-)$ . p $\Delta 54-98$  was derived from Fp $\Delta 13\text{T}1\text{F}1(-)$  by digesting with *Bst*81, filling in with T4 DNA polymerase, digesting with *Hind*III and religating.



### Sequences necessary for cyclin degradation

The protein synthesis requirement of the early *Xenopus* cell cycles can be fulfilled by the translation of sea urchin cyclin B messenger RNA<sup>10</sup>. Translation of this mRNA drives both entry into and exit from mitosis. In cells and extracts that transit through the cell cycle, cyclin degradation is transient, making a detailed biochemical analysis of the mechanism and regulation of cyclin degradation difficult. However, a truncated cyclin protein, cycΔ90, that lacks the first 90 amino acids of cyclin, is not degraded at mitosis but is able to activate MPF (ref. 3). In the presence of this mutant a persistent metaphase state is established in which exogenously added wild-type cyclins are degraded<sup>3</sup>. We have made use of the stable mitotic state induced by cycΔ90 to dissect the cyclin destruction pathway and characterize the domain of the cyclin molecule that confers cell-cycle-regulated proteolysis.

To prepare stable mitotically-arrested extracts we added bacterially expressed cycΔ90 to interphase *Xenopus* cell cycle extracts. Figure 1 shows the effect of adding cycΔ90 to these interphase extracts. Histone H1 kinase activity, a measure of MPF activity, peaked 30 minutes after addition of cycΔ90 and remained at high levels for the duration of the experiment. Cyclin proteolysis was first detectable between 40 and 50 minutes after the addition of cycΔ90. Cyclin added at later times was also degraded, showing that cyclin proteolysis activity is maintained at high levels for at least an hour. In the absence of added cycΔ90 protein, the extracts remained in interphase where H1 kinase activity was low and cyclin was stable. Thus interphase extracts activated by cycΔ90 generate a constitutively active cyclin proteolytic activity. We refer to these cycΔ90 extracts as mitotically arrested extracts.

We have previously shown that amino acids 13–90 of sea urchin cyclin B are essential for cell-cycle-regulated destruction<sup>3</sup>. We asked whether the N-terminal region of the molecule was sufficient to confer M-phase-specific degradation to another protein. We used the heterologous sea urchin cyclin protein for these studies for reasons of convenience; all discussion of specific sequences and positions refer to this cyclin. We constructed a hybrid protein, 13–91prA, which consists of amino acids 13–91 of sea urchin cyclin B fused to a region of *Staphylococcus aureus* protein A that binds IgG. This fusion protein was

expressed in *Escherichia coli*, purified by affinity chromatography and radio-iodinated. The 13–91prA protein was efficiently degraded in mitotic extracts but was stable in interphase extracts (Fig. 2c, d), establishing that residues 13–91 of cyclin contain sequences that are sufficient for cell-cycle-regulated proteolysis.

When we compared the N-terminal 90 amino acids of 13 cyclins from eight species we found only 10–20% sequence conservation. However, two features stood out: first, there is a 9-amino-acid region, RAALGNISN (single-letter amino-acid code), the 'destruction box', which contains two invariant residues, four highly conserved residues, and one residue (position 47) that serves to differentiate A and B type cyclins as it is always asparagine, aspartic acid or glutamic acid in B-type cyclins, and always valine in A-type cyclins (see Fig. 2f). In addition, there are three positions where there is weak conservation of a two- or three-amino-acid motif, whose functional significance is unclear. Second, we noted that although residues 54–90 show virtually no conservation across all species analysed, there is a pronounced enrichment for lysine residues. The number of lysine residues in this region ranges from 1 to 13, with a median of six.

To test whether these structural elements have functional significance, the proteolysis of additional cyclin mutants was analysed. Plasmids encoding mutant cyclins were transcribed *in vitro* and translated in a reticulocyte lysate system and the translation products were added to mitotic or interphase extracts. Deleting residues 66–98 from cycΔ13, a derivative of cyclin that lacks the first 13 amino acids, produced a derivative that contained the destruction box, the isolated small motifs, and a portion of the lysine-rich region. This derivative was degraded in mitotic extracts and stable in interphase extracts (Fig. 2a, b). By contrast, a slightly larger deletion spanning residues 54–98 produced a derivative stable in both mitotic and interphase extracts (Fig. 2a, b, g). Similarly, using *E. coli* expressed proteins, we found that fusing residues 13–66 to protein A was sufficient to confer cell-cycle-regulated proteolysis on protein A, but residues 13–53 were insufficient (Fig. 2c, d). The results suggest that residues 54–66 contain a determinant required for cyclin proteolysis. Furthermore, deletion of residues 1–53 blocked degradation in mitotic extracts (data not shown), suggesting a role for the destruction box in cyclin proteolysis. To test this, we converted the invariant arginine at position 42 of cycΔ13 to a cysteine to create the derivative cycΔ13-R42C. Figure 2a, c shows that this mutant protein cannot be degraded, and that the same mutation in a cyclin-protein A fusion also blocks degradation. Figure 2g summarizes the behaviour of the mutant constructs.

To explore further the relationship between cyclin proteolysis and exit from mitosis we tested the ability of cycΔ13-R42C to drive extracts in and out of mitosis by adding cycΔ13-R42C mRNA to nuclease-treated cell-cycle extracts containing sperm nuclei. Accumulation of cycΔ13-R42C, as well as the control cyclin cycΔ13, activated histone H1 kinase (Fig. 3) and caused breakdown of the nuclear envelope and chromosome condensation. In the cycΔ13-containing extract, cyclin degradation was induced 40 min after the beginning of the reaction and H1 kinase activity fell shortly afterwards. By contrast, in the cycΔ13-R42C-containing extract histone H1 kinase and cyclin protein levels stayed high, and nuclei remained in mitosis, supporting the argument that cyclin destruction is essential for exit from mitosis.

### Cyclin is conjugated to ubiquitin

When we analysed long exposures of the gels of the labelled cycΔ13 protein during the experiment described above, we noticed that just before the onset of cyclin degradation a small amount of cyclin was apparently converted to a faint ladder of higher  $M_r$  forms, which disappeared with destruction of the unmodified cyclin (see Fig. 3). These forms were not observed with the non-degradable cycΔ13-R42C. Because the average difference in  $M_r$  between the bands forming this ladder was

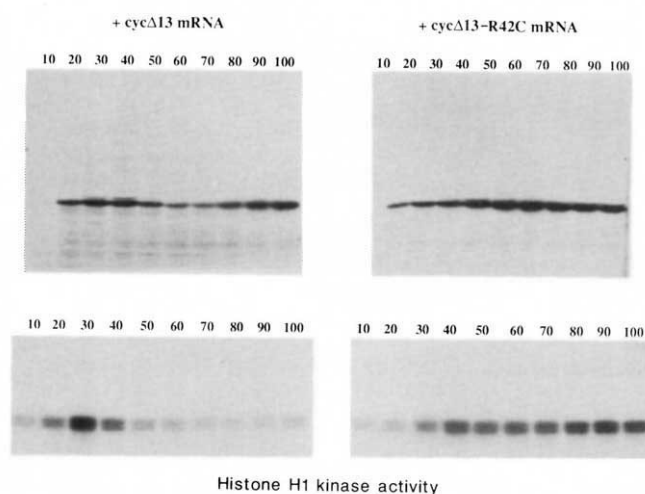


FIG. 3 CycΔ13-R42C causes metaphase arrest and a degradation intermediate is detectable during the degradation of cycΔ13. Activated cycling extracts, containing <sup>35</sup>S-methionine, were programmed with mRNA for cycΔ13 or cycΔ13-R42C and at the indicated times samples were removed for analysis of <sup>35</sup>S-labelled proteins and histone H1 kinase activity. METHODS. Extracts were prepared and nuclease-treated as described<sup>16</sup>. mRNA was generated by *in vitro* transcription of FpΔ13TF1(–) and pΔ13-R42C (see Fig. 2). <sup>35</sup>S-labelled proteins and histone H1 kinase activity were analysed as described in Fig. 1.



~7K, the  $M_r$  of ubiquitin, we asked whether these bands were conjugates of cyclin and ubiquitin. We first sought a more sensitive means for visualizing the putative cyclin-ubiquitin conjugates. The cyclin-protein A fusion protein, 13-91prA, could be radio-iodinated to high specific activity and showed cell-cycle-specific degradation. As shown in Fig. 4a, when the labelled 13-91prA was added to mitotic extracts and samples removed at various times during degradation, a ladder of high  $M_r$  forms with a spacing of ~7K was visible on overexposed gels. These forms decreased in amount as the cyclin-protein A product was degraded. As the only labelled protein in the extract was the iodinated 13-91prA and lower  $M_r$  degradation products derived from it, the high  $M_r$  ladder must have derived from the cyclin-protein A fusion. To enhance our ability to follow these

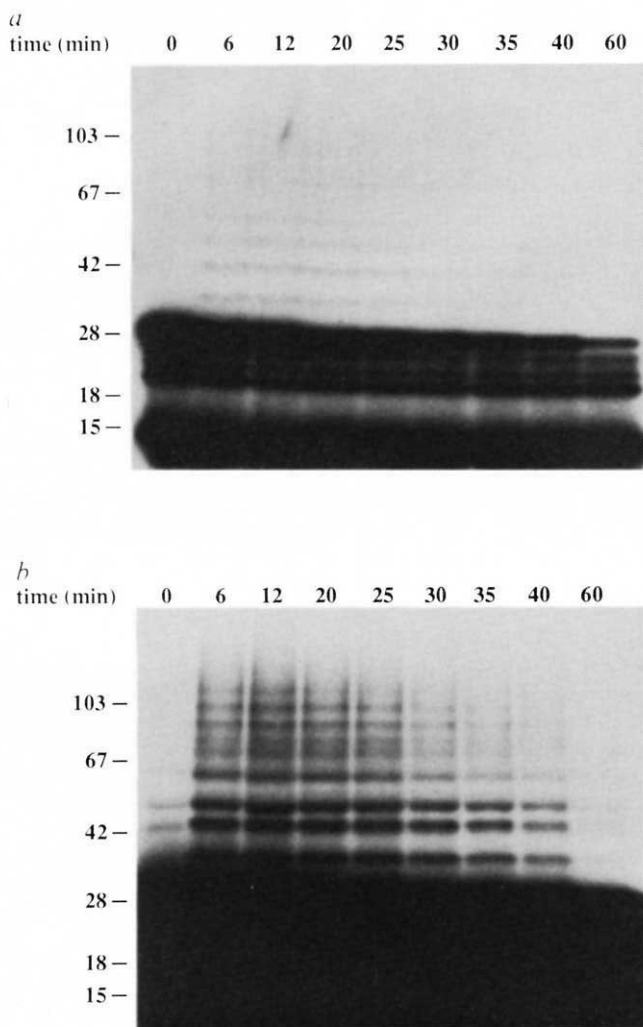


FIG. 4 Kinetic analysis of degradation of 13-91prA and of the abundance of high- $M_r$  forms of 13-91prA. Labelled 13-91prA was added to mitotic extracts. At the indicated times samples were quenched with sample buffer (a) or precipitated with IgG-Sepharose (b), and analysed by SDS-PAGE and autoradiography.

**METHODS.** Extracts and proteins were prepared as described in Fig. 2.  $^{125}$ I-labelled 13-91prA was added to mitotic extracts at 5,000 c.p.m. per ml. 0.5 ml samples were quenched with sample buffer and electrophoresed directly, or 10 ml samples were quenched by addition of 10 volumes RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 10 mM *N*-ethylmaleimide. The solution was incubated for 10 min on ice before cysteine was added to 0.1%. IgG-Sepharose beads were added and incubated for 1 h at 4 °C on a rotating wheel. Beads were pelleted by centrifugation and washed three times in RIPA buffer. The precipitated proteins were recovered by boiling for 2 min in sample buffer before analysis by SDS-PAGE and autoradiography.

forms we purified and concentrated 13-19prA and its complexes using the protein A epitope tag and IgG-Sepharose; under these conditions the high  $M_r$  ladder is easily detected (Fig. 4b).

To ascertain whether these high  $M_r$  forms were indeed ubiquitin adducts to cyclin, we added radio-iodinated ubiquitin to interphase and mitotic extracts in the presence or absence of 13-91prA. After a 10-min incubation the 13-91prA and any conjugated products were affinity-purified with IgG-Sepharose beads. An autoradiograph of the affinity-purified material is shown in Fig. 5. In mitotic extracts, a ladder of labelled bands was precipitated only when 13-91prA was present (compare lanes 3 and 4). These bands precisely coincided with the ladder of bands generated from  $^{125}$ I-labelled 13-91prA in mitotic extracts (compare lanes 4 and 5). Precipitation of  $^{125}$ I-labelled ubiquitin conjugated to 13-91prA and production of slowly migrating forms of [ $^{125}$ I] 13-91prA were also detected in interphase extracts (lanes 2 and 6; see weak bands at 31 and 38K), but at very much reduced levels. These experiments show unambiguously that the high  $M_r$  products formed from 13-91prA in mitotic extracts contain ubiquitin and most likely represent multi-ubiquitinated 13-91prA, and that the conjugation of ubiquitin to cyclin derivatives is regulated during the cell cycle. When a similar experiment was performed with the protein A fusion protein containing the arginine to cysteine mutation, 13-91prA-R42C, some of the ubiquitinated forms (31 and 38K) appear specifically in mitosis (lane 11) but none of the higher, multi-ubiquitinated forms were observed (Fig. 5, lanes 11-12). Thus a cyclin derivative that cannot be degraded cannot be multi-ubiquitinated, supporting the hypothesis that multi-ubiquitination of cyclin is required for its destruction.

### Kinetics of cyclin ubiquitination

The ubiquitinated cyclin adducts could represent intermediates on the pathway of cyclin destruction in mitosis or non-productive side-products that accumulate in mitosis while cyclin is being degraded by a different pathway. As no specific inhibitors of ubiquitin conjugation or of the protease system that degrades ubiquitinated proteins are known, we could not demonstrate directly that the ubiquitin-cyclin conjugates are intermediates in cyclin degradation. Therefore, we attempted to demonstrate that at steady state the flux of cyclin through the ubiquitin-containing intermediates is equal to the overall rate of cyclin degradation.

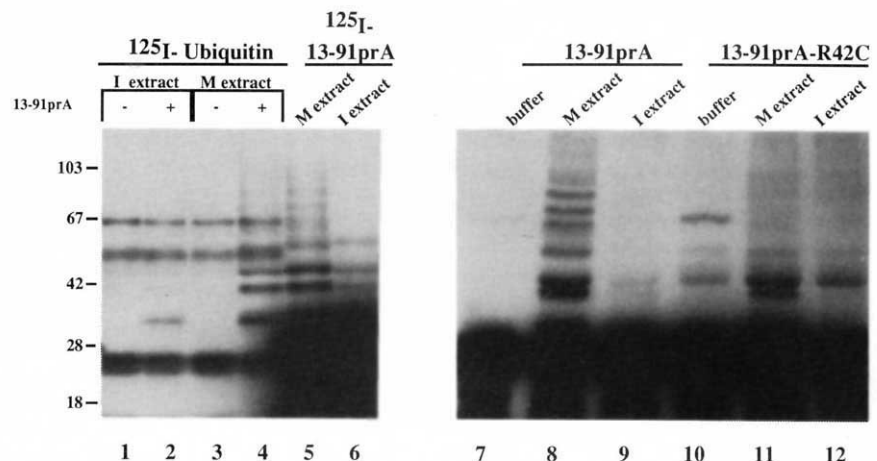
To model the kinetics of cyclin degradation we first estimated the percentage of 13-91prA present as ubiquitin conjugates during the course of degradation in mitosis. From the time course in Fig. 4 we estimate that ~3% of the input protein is present as conjugates between 0 and 35 min. We determined the half-life of these conjugates by a 'pulse-chase' experiment. Radiolabelled 13-91prA was allowed to accumulate into ubiquitin-13-91prA conjugates in a mitotically arrested extract. After 10 min unlabelled 13-91prA was added at levels that saturated the cyclin destruction pathway and the time course of the loss of radiolabelled ubiquitinated conjugates was analysed by gel electrophoresis. Radioactivity in the entire collection of ubiquitin conjugates decayed to less than half the original amount within 120 s (Fig. 6a). An exponential curve was fitted to data representing the concentration of total labelled ubiquitin conjugates with time and from this the half-life of the conjugates was determined to be 90 s.

Assuming that the ubiquitination and subsequent destruction reactions follow first order kinetics, the concentration of ubiquitin intermediates,  $I(t)$ , is given by the equation

$$I(t) = k_1 A(t) - k_2 I(t)$$

where  $A(t)$  represents the concentration of the unconjugated cyclin, and  $k_1$  and  $k_2$  are first-order rate constants. The value of  $k_2$  was estimated from the pulse-chase experiment. Because the intermediates do not accumulate appreciably, we argue that the initial conjugation step must be rate limiting. Thus  $k_1$  is

FIG. 5 The high- $M_r$  forms of cyclin are ubiquitin conjugates. Lanes 1–4 contain IgG-binding proteins labelled with  $^{125}\text{I}$ -labelled ubiquitin that are generated in interphase (lanes 1–2) or mitotic (lanes 3–4) extracts in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of unlabelled 13–91prA. Lanes 5–8 contain the IgG-binding proteins generated from  $^{125}\text{I}$ -13–91prA (lanes 5–6) or  $^{125}\text{I}$ -13–91prA-R42C (lanes 7–8) in mitotic (lanes 5 and 7) and interphase (lanes 6 and 8) extracts. **METHODS.** Ubiquitin was purchased from Sigma. Proteins were radiolabelled and extracts prepared as described in Fig. 2. Lanes 1–4, ubiquitin (105 c.p.m. per mg) was added to extracts at a concentration of  $2 \times 10^4$  c.p.m. per ml, and incubated 10 min. To lanes 2 and 4,  $3 \mu\text{g}$  of 13–91prA was added, incubated 10 min, and the reaction stopped as described in Fig. 4. In lanes 5–8,  $5 \times 10^4$  c.p.m. of labelled 13–91prA or 13–91prA-R42C (both at  $10^6$  c.p.m. per mg) was added to 10 ml of extracts and incubated for 10 min then the reaction was quenched. Proteins that bind to IgG-Sepharose were recovered and analysed as described in Fig. 4.



roughly equal to the overall reaction rate, which was estimated by cutting out the bands containing the unmodified cyclin from the gel shown in Fig. 4b, counting them, and fitting the data to an exponential decay function. Experimental values of  $I(t)$  were determined by cutting out the regions of the gel that contain the modified forms of cyclin from Fig. 4b and counting them. Figure 6b compares the values of concentration of unconjugated cyclin and cyclin-ubiquitin conjugates measured from Fig. 4b with the values predicted by mathematical simulation using the derived values of  $k_1$ , and  $k_2$ . The close agreement strongly suggests that all of the cyclin that is degraded passes through a ubiquitinated intermediate.

## Discussion

Progression through the cell cycle depends on the specific proteolysis of cyclin at the end of mitosis. To study the mechanism of cyclin degradation we have generated a *Xenopus* extract arrested in metaphase that constitutively degrades cyclin. Our system avoids the complications of standard cell-cycle extracts that only degrade cyclin transiently. We used this extract to map the sequences that confer cell-cycle-regulated degradation upon cyclin and to show that cyclin-ubiquitin conjugates appear during cyclin degradation. These conjugates accumulate and disappear with kinetics that are consistent with their being intermediates in the course of cyclin degradation, and do not form with cyclin derivatives that cannot be degraded. This evidence strongly suggests that the cell-cycle-regulated degradation of cyclin occurs by way of the ubiquitin pathway.

An N-terminal domain of cyclin is sufficient to confer cell-cycle-regulated degradation. With the demonstration that cyc $\Delta$ 90 cannot be degraded, this shows that the N terminus of cyclin is necessary and sufficient for cyclin degradation. By fusing this domain to protein A we created a hybrid protein that could be easily recovered from reactions by virtue of its binding to IgG. An isolated domain of cyclin, consisting of residues 13–91, which is entirely separate from the domain required for the activation of p34<sup>cdc2</sup> (ref. 3), can also be degraded in a cell-cycle-regulated fashion (data not shown). The region between amino acids 13 and 66 is sufficient to confer mitotic-specific degradation to the C-terminal portion of the cyclin molecule or to a foreign protein, protein A. Within this region there are two conserved features. The first is a sequence of nine amino acids (42–51) that we term the destruction box. Mutation of the invariant arginine residue at position 42 prevents the degradation of cyclin or a cyclin–protein A fusion carrying the mutation. We suggest that this region is recognized by some

component of the ubiquitin-conjugating system. Although the destruction boxes of A and B type cyclins are similar, at one position there is a difference between A and B cyclins which may correlate with the earlier degradation of the A cyclins found in many organisms<sup>17–20</sup>. One of the *Saccharomyces cerevisiae* G1 cyclins, CLN2<sup>23</sup>, contains the sequence RMGLVINAK, which has weak homology to the destruction box. The G1 cyclins have been identified only in budding yeast and though they are unstable it is not clear whether their stability is regulated during the cell cycle. Similarly, the yeast transcriptional regulator  $\alpha$ 2 has the motif RDILVFLSR<sup>21</sup> in a domain involved in destabilizing the protein<sup>22</sup>. The significance of these weak homologies to the destruction box remains to be established.

Sequences outside the destruction box are also required for cyclin degradation. Deletion derivatives of cyclin and of cyclin–protein A fusions that lack residues 54–66 are stable. In sea urchin cyclin B, residues 54–66 contain 4 lysine residues. The region between residues 51–90 is not conserved among cyclins, but is generally rich in lysine (17% averaged over all cyclin sequences). Gonda and coworkers showed that the ubiquitin-mediated destruction of proteins whose N-terminal amino acid is recognized by ubiquitin-conjugating enzymes requires a nearby lysine residue to act as an acceptor site for ubiquitin conjugation<sup>11,24,25</sup>. We speculate that the lysine residues C-terminal to the destruction box are the sites for ubiquitin conjugation. Interestingly, cyclins from many organisms show cell-cycle-dependent degradation in the *Xenopus* extracts (our unpublished results). This suggests that there is a minimal structural requirement for degradation.

Though cyclin and proteins containing cyclin peptides are clearly ubiquitinated, the ubiquitinated conjugates might not be intermediates in the pathway of cyclin degradation at mitosis. Although ubiquitin-mediated degradation has been well-studied in *in vitro* systems (for example, RNase A, casein, bovine serum albumin and lysozyme<sup>26</sup>), many of the substrates have been denatured or chemically-modified proteins and it is possible that the ubiquitin pathway serves principally to target misfolded proteins for destruction. Several properties of the ubiquitination of cyclin suggest that it does indeed represent the pathway of destruction. First, the observed ubiquitination of cyclin shows a tight dependence on the cell cycle. In mitotic extracts, 3% of 13–91prA is found as ubiquitin conjugates; in interphase extracts the level of ubiquitination of this protein is reduced about 10-fold. Second, a non-degradable mutant cyclin, cyc $\Delta$ 13-R42C, is not detectably multi-ubiquitinated under conditions where cyclin multi-ubiquitination was easily detected. A similar mutant



in the cyclin-protein A conjugate also blocks both degradation and ubiquitination. Third, the kinetics of the ubiquitination and degradation reactions are consistent with a requirement that all of the cyclin must pass through ubiquitin conjugates before degradation and are inconsistent with a model in which only a small amount of degradation occurs through ubiquitin and the majority by a different pathway. Figure 6b shows experimentally determined levels of ubiquitin conjugates and cyclin protein at various times fitted to a model for cyclin degradation in which ubiquitination of cyclin is a prerequisite for its degradation.

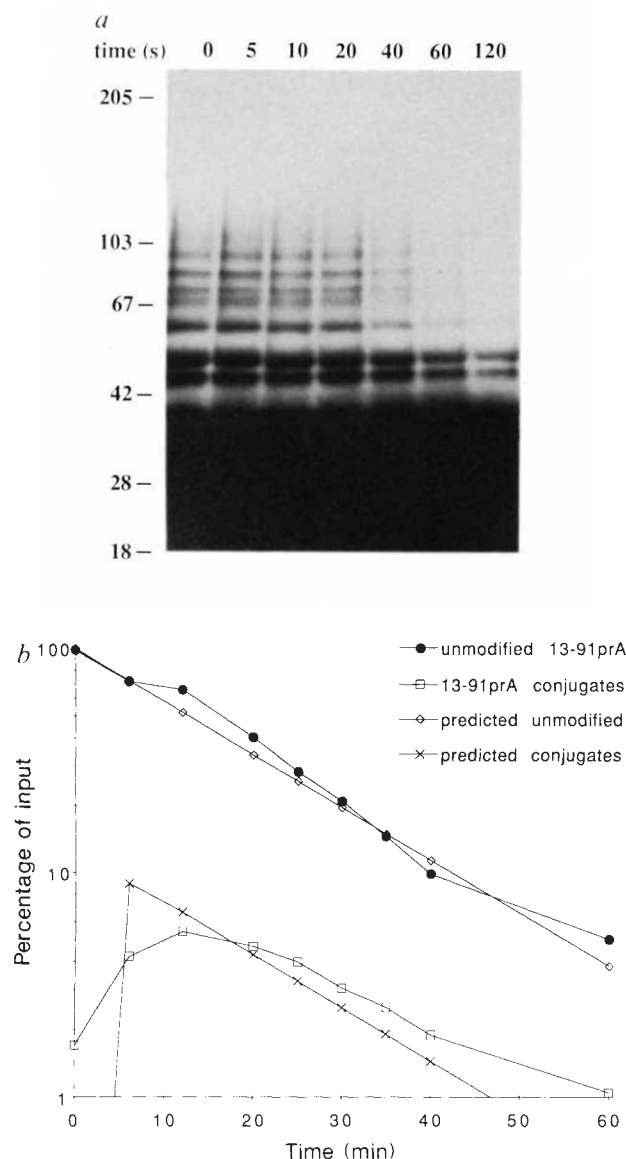


FIG. 6 Pulse-chase analysis of the stability of the ubiquitin conjugates. *a*, <sup>125</sup>I-labelled 13-91prA was added to a mitotically arrested extract. After 10 min, aliquots were removed and added to a 16.5× mass excess of unlabelled 13-91prA. At the indicated times the reaction was quenched and the IgG-binding species were analysed as described in Fig. 4. The decrease in radioactivity in the conjugates was quantitated by  $\gamma$  counting, plotted as a function of time and fitted to an exponential decay function to give  $k_2 = 0.46 \text{ min}^{-1}$  ( $R = 0.97$ ), corresponding to a half-life of 1.5 min. *b*, The amount of modified and unmodified cyclin in the experiment shown in Fig. 4b plotted and compared with the values predicted from the mathematical model described. The value used for  $k_1$  in the model was  $0.053 \text{ min}^{-1}$  ( $R = 0.99$ ), which corresponds to  $t_{1/2} = 13 \text{ min}$ .

METHODS. Extracts and 13-91prA were prepared as described in Fig. 2, and the proteins were recovered and analysed as Fig. 4. The modelling was performed using the Stella software package.

Given the measured rate of degradation of cyclin, the measured half-life of the ubiquitin-cyclin conjugates and a reaction sequence in which the ubiquitin conjugates are intermediates in the destruction pathway, this model yields a level of ubiquitin conjugates of cyclin found during the degradation reaction that is almost exactly equal to that observed.

As the ubiquitin pathway seems to be responsible for cyclin proteolysis in mitosis, some aspect of the recognition of cyclin by the ubiquitin-conjugating system must be regulated by MPF. Several possibilities exist. MPF could activate a cyclin-specific enzyme that is directly involved in conjugating ubiquitin to cyclin. Several forms of ubiquitin-conjugating enzymes have been characterized<sup>10,27</sup>, so it is conceivable that there is a form of these enzymes specific for cyclin. Alternatively, the ubiquitin-conjugating enzymes may be active throughout the cell cycle and MPF might stimulate an activity that makes cyclin a better substrate for ubiquitination. Examples of such activities are protein kinases that phosphorylate cyclin; cyclin-binding proteins that interact with the ubiquitination machinery; or a cyclin-specific endoprotease, activated by MPF, that could cleave cyclin and expose a destabilizing amino terminus<sup>28</sup>. Although, we cannot so far distinguish between these possibilities, the biochemical system we have established should permit the fractionation and characterization of the ubiquitin-conjugating activity and the identification of those activities that are triggered by MPF. There is one other known example of a specific protein of physiological interest that shows a regulated degradation by the ubiquitin pathway: the light-activated degradation of phytochrome in plants<sup>15,29</sup> and this system has interesting parallels with cyclin. In both cases the protein whose degradation is regulated accumulates under one set of conditions and is degraded under another. Active molecules are generated when light interacts with phytochrome or p34<sup>cdc2</sup> associates with cyclin and undergoes specific post-translational modifications. The duration of the physiological effects of the activated molecule is limited because it induces its own ubiquitin-mediated degradation. In both systems neither the identity of the ubiquitin conjugating enzyme nor the nature of the event that allows recognition of the substrate by the ubiquitin conjugating system is known. However, there are a number of mutants in ubiquitin-conjugating enzymes that lead to interesting phenotypes including cell cycle arrest<sup>12,13,30-34</sup>. Of particular interest is the result that mutations in a specific ubiquitin ligase in budding yeast, *CDC34*, lead to arrest of the cell cycle at the G1 to S transition<sup>13</sup>, an arrest which could be due to a failure to degrade one or more of the G1 cyclins.

Cyclin degradation at the metaphase-anaphase transition in the mitotic cycles of the early frog embryo is not regulated by the state of the mitotic spindle<sup>35,36</sup>. However, in many other cells the metaphase to anaphase transition is tightly regulated by feedback controls that prevent the onset of anaphase until the mitotic spindle has been fully assembled. In most cells the molecules that mediate the feedback control are not known. One exception is unfertilized frog eggs, which are arrested in metaphase of meiosis II by an activity named cytoskeletal factor (CSF), one of whose components is the *c-mos* proto-oncogene<sup>37</sup>. In CSF-arrested cells, cyclin is stable even though MPF levels are high, suggesting that the key event regulating the onset of anaphase may be the degradation of cyclin<sup>3</sup>. The experiments reported here suggest that the degradation of cyclin is a consequence of its recognition by the ubiquitin-conjugating system. An understanding of this recognition step may be required for a complete understanding of the mechanisms regulating the completion of mitosis and meiosis. □

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## LETTERS TO NATURE

## Evidence for a common central-engine mechanism in all extragalactic radio sources

Steve Rawlings & Richard Saunders

Mullard Radio Astronomy Observatory, Cavendish Laboratory,  
Madingley Road, Cambridge CB3 0HE, UK

**EXTRAGALACTIC radio sources produce radio waves and narrow emission lines by very different physical processes: synchrotron radio emission arises from lobes filled with magnetized plasma extending over scales of kiloparsecs to megaparsecs, fed by the total kinetic power,  $Q$ , of jets driven by a central engine, whereas narrow-line luminosity  $L_{\text{NLR}}$  arises from gas, typically concentrated in the inner few kiloparsecs, that has been photoionized by a nuclear source. We report here the discovery of a close relationship between  $Q$  and  $L_{\text{NLR}}$ —an approximate proportionality which extends over four orders of magnitude from low- $Q$  radio sources with relaxed structures to high- $Q$ , radio-luminous classical double-lobe radio galaxies. Objects with broad Balmer lines follow the same trend as those without, showing that quasar-like photoionizing sources are ubiquitous but not always obvious. Moreover, all radio-source central engines channel at least as much power into the jets as is radiated by accretion: this high efficiency implies that the engine is a massive spinning black hole which both powers the jets and controls the accretion rate.**

We have recently found a positive correlation between the narrow-line luminosities and the radio luminosities of an unbiased sample of low-redshift ( $z < 0.5$ ) Fanaroff and Riley<sup>1</sup> (hereafter FR) class II radio galaxies<sup>2</sup>, providing quantitative evidence that the production of nuclear optical line emission and the production of large-scale radio emission are somehow intrinsically linked. The radio luminosity,  $L_{\text{rad}}$ , of an extended extragalactic radio source is much smaller than the bulk kinetic power  $Q$  that goes into increasing the energy (stored in fields and particles) of the growing lobes and pushing back the external medium<sup>3</sup>. Because  $Q$  is a much more direct measure than  $L_{\text{rad}}$  of what powers the radio source, and as some scatter in the relationship between  $L_{\text{rad}}$  and  $L_{\text{NLR}}$  may be induced simply by the effects of the lobe environment, we investigate here the observational relation between  $Q$  and  $L_{\text{NLR}}$ .

For a total lobe energy  $E$ , an efficiency  $\eta$  that allows for work

done on the external medium, and a lobe age  $T$ , the power  $Q = E/T\eta$ . We first take  $E$  as the equipartition energy<sup>4</sup>. (The main assumption here is that the electrons and the magnetic field make an equal contribution to the total energy density, which corresponds closely to the minimum value required to produce the observed synchrotron emission; this energy density is integrated over the volume of the radio source to produce the equipartition energy.) We take  $\eta$  to be 0.5, a value suggested by identifying compact hotspots as the working surfaces of relativistic jets<sup>5</sup>. We can thus evaluate  $Q$  for a source if  $T$  is known. Spectral ages are available for a number of radio galaxies; these are derived using standard formulae that relate the curvature of radio spectra to the energy losses incurred by relativistic electrons because of their own synchrotron radiation and because they scatter microwave-background photons by the inverse Compton process<sup>6</sup>. Where spectral ages are unavailable, we can estimate the age as follows. For a lobe of length  $D/2$ , width  $W$  and growth speed  $V = D/2T$ , we can relate the equipartition pressure  $p_{\text{eq}}$  in the lobe to the confining density  $\rho$  by  $\rho V^2 = kD^2 W^{-2} p_{\text{eq}}$ ;  $k$  reflects how the lobes are confined.  $D$ ,  $W$  and  $p_{\text{eq}}$  are all straightforward to measure. For  $k = 25$  there is good agreement between the estimated and spectral ages of the 16 FR II radio galaxies for which both have been reliably measured<sup>7</sup>, and we therefore adopt this value. Sufficiently accurate estimates of  $\rho$  can be made from X-ray and optical data<sup>5</sup>.

We have evaluated  $Q$  for the 39 FR II radio galaxies in our unbiased sample of ref. 2; details of the assumptions made and the data used are given in Table 1. In Fig. 1 we plot  $Q$  against  $L_{\text{NLR}}$ ; the method used to calculate  $L_{\text{NLR}}$  from spectrophotometric data is described in the caption. There is a strong positive correlation extending over roughly two orders of magnitude in each variable, which is significantly better than the correlation between  $L_{\text{rad}}$  and  $L_{\text{NLR}}$  (ref. 2). Our unbiased sample was drawn from the complete 3CR sample<sup>8</sup>, but our selection criteria excluded three classes of radio source: (1) those of FR class I; (2) those associated with quasars rather than galaxies; (3) all objects at  $z > 0.5$ . To include these in our investigation, we also plot those 3CR objects in each class for which there are suitable data to calculate  $Q$  and  $L_{\text{NLR}}$ . For the FRIs, values of  $E$  and spectral ages were used as above; for the quasars and high- $z$  radio galaxies, growth speeds of  $0.1c$  were assumed based on existing values of  $T$  for similar objects (for example, ref. 6). With the addition of these objects,  $Q$  and  $L_{\text{NLR}}$  are correlated over approximately four orders of magnitude in each variable with scatter of only one order of magnitude; the best-fit line

TABLE 1 Data for calculation of jet power

a, Unbiased sample: 39 FR II radio galaxies with  $z < 0.5$ 

| Name    | $T$ (yr)        | $n_e$ ( $m^{-3}$ ) | $Q$ (W)            | Name    | $T$ (yr)        | $n_e$ ( $m^{-3}$ ) | $Q$ (W)            |
|---------|-----------------|--------------------|--------------------|---------|-----------------|--------------------|--------------------|
| 3C33    |                 | $5 \times 10^1$    | $9 \times 10^{37}$ | 3C284   | $3 \times 10^7$ |                    | $1 \times 10^{38}$ |
| 3C35    | $1 \times 10^8$ |                    | $1 \times 10^{37}$ | 3C285   | $4 \times 10^7$ |                    | $1 \times 10^{37}$ |
| 3C42    |                 | $5 \times 10^2$    | $3 \times 10^{38}$ | 3C295   |                 | $1 \times 10^4$    | $7 \times 10^{38}$ |
| 3C46    |                 | $1 \times 10^2$    | $4 \times 10^{38}$ | 3C300   | $3 \times 10^7$ |                    | $1 \times 10^{38}$ |
| 3C67    |                 | $1 \times 10^4$    | $2 \times 10^{38}$ | 3C303   |                 | $5 \times 10^2$    | $5 \times 10^{37}$ |
| 3C79    | $6 \times 10^6$ |                    | $6 \times 10^{38}$ | 3C319   | $3 \times 10^7$ |                    | $4 \times 10^{37}$ |
| 3C98    |                 | $5 \times 10^1$    | $3 \times 10^{37}$ | 3C321   |                 | $1 \times 10^1$    | $1 \times 10^{38}$ |
| 3C109   |                 | $5 \times 10^1$    | $6 \times 10^{38}$ | 3C326   |                 | $1 \times 10^0$    | $1 \times 10^{38}$ |
| 4C14.11 |                 | $1 \times 10^3$    | $5 \times 10^{37}$ | 3C341   |                 | $1 \times 10^1$    | $2 \times 10^{39}$ |
| 3C123   |                 | $1 \times 10^3$    | $5 \times 10^{38}$ | 3C349   |                 | $5 \times 10^1$    | $2 \times 10^{38}$ |
| 3C173.1 |                 | $1 \times 10^2$    | $5 \times 10^{38}$ | 3C381   |                 | $1 \times 10^2$    | $2 \times 10^{38}$ |
| 3C184.1 | $3 \times 10^7$ |                    | $4 \times 10^{37}$ | 3C382   |                 | $5 \times 10^1$    | $3 \times 10^{37}$ |
| DA240   |                 | $5 \times 10^0$    | $1 \times 10^{37}$ | 3C388   |                 | $1 \times 10^3$    | $3 \times 10^{37}$ |
| 3C192   |                 | $5 \times 10^1$    | $5 \times 10^{37}$ | 3C390.3 |                 | $1 \times 10^2$    | $5 \times 10^{37}$ |
| 3C219   | $6 \times 10^7$ |                    | $1 \times 10^{38}$ | 3C401   |                 | $1 \times 10^3$    | $1 \times 10^{38}$ |
| 3C223   |                 | $1 \times 10^1$    | $2 \times 10^{38}$ | 3C436   | $2 \times 10^7$ |                    | $9 \times 10^{37}$ |
| 4C73.08 | $3 \times 10^7$ |                    | $6 \times 10^{37}$ | 3C438   |                 | $5 \times 10^3$    | $3 \times 10^{38}$ |
| 3C234   | $5 \times 10^6$ |                    | $3 \times 10^{38}$ | 3C452   | $3 \times 10^7$ |                    | $6 \times 10^{37}$ |
| 3C236   | $8 \times 10^7$ |                    | $1 \times 10^{38}$ | 3C457   |                 | $1 \times 10^1$    | $1 \times 10^{39}$ |
| 3C244.1 | $7 \times 10^6$ |                    | $3 \times 10^{38}$ |         |                 |                    |                    |

b, 3CR FRIs with 178-MHz radio luminosity  $< 2 \times 10^{25} \text{ W Hz}^{-1} \text{ sr}^{-1}$ 

|        |                 |                    |         |                 |                    |
|--------|-----------------|--------------------|---------|-----------------|--------------------|
| 3C31   | $4 \times 10^8$ | $8 \times 10^{35}$ | 3C338   | $3 \times 10^7$ | $1 \times 10^{36}$ |
| 3C66B  | $3 \times 10^7$ | $2 \times 10^{36}$ | NGC6251 | $1 \times 10^8$ | $5 \times 10^{36}$ |
| 3C76.1 | $8 \times 10^7$ | $1 \times 10^{36}$ | 3C465   | $2 \times 10^8$ | $5 \times 10^{36}$ |
| 3C264  | $1 \times 10^7$ | $3 \times 10^{36}$ |         |                 |                    |

Equipartition energy was calculated assuming no energy in protons, minimum and maximum frequencies of 10 MHz and 10 GHz, and a volume-filling factor of one. Spectral ages  $T$ , where available, were taken from the literature (refs 6, 7, 14 for the FR IIs and refs 15–21 for the FR Is); confining electron number densities  $n_e$  were estimated, where required, using the available X-ray and optical data<sup>5,7</sup> and were related to gas density  $\rho$  by  $\rho = 1.4 n_e m_p$  ( $m_p$  is the proton rest mass). High- $z$  FR II radio sources studied were 9 3CR quasars with  $z < 1$  (3C47, 3C175, 3C196, 3C215, 3C249.1, 3C263, 3C275.1, 3C334, 3C351) and 24 3CR radio galaxies (3C6.1, 3C22, 3C34, 3C55, 3C172, 3C175.1, 3C184, 3C217, 3C226, 3C228, 3C247, 3C263.1, 3C265, 3C268.1, 3C277.2, 3C280, 3C289, 3C330, 3C337, 3C340, 3C343.1, 3C352, 3C427.1, 3C441). The references for the radio data for all objects are given in ref. 8. We take the Hubble constant  $H_0$  to be  $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ , and the cosmological density parameter  $\Omega_0$  and the cosmological constant  $\Lambda$  to be zero.

$Q \propto L_{\text{NLR}}^{0.9 \pm 0.2}$  implies that the relationship is close to a proportionality.

A striking feature of Fig. 1 is that quasars and broad-line radio galaxies follow the general trend defined by the narrow-line radio galaxies (NLRGs), despite the vast differences in their continua. This is important evidence supporting models in which

NLRGs have central quasars hidden from us by obscuration but not hidden from their associated narrow-line regions<sup>9,10</sup>. We note that quasars may appear to have higher  $L_{\text{NLR}}$  than NLRGs with similar  $L_{\text{rad}}$  (ref. 11) because objects likely to be classified as quasars will be biased to higher photoionizing luminosity  $L_{\text{phot}}$  and thus higher  $L_{\text{NLR}}$ , and the combination of  $Q$ – $L_{\text{NLR}}$

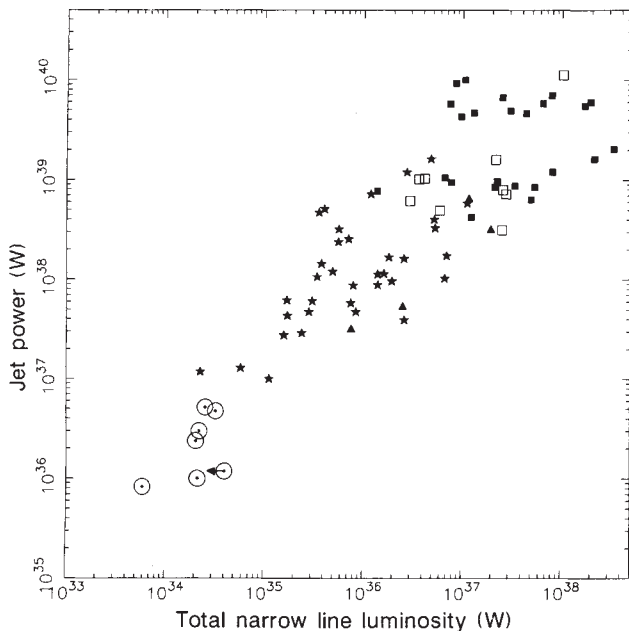


FIG. 1 Bulk kinetic power of jet  $Q$  plotted against total narrow-line luminosity  $L_{\text{NLR}}$  for the radio sources in Table 1. ○, FR I sources; ★, FR II radio galaxies in our unbiased sample of objects with  $z < 0.5$ ; ▲, broad-line radio galaxies; ■, radio galaxies with  $0.5 < z < 1$ ; □, quasars with  $z < 1$ .  $L_{\text{NLR}}$  has been measured for all objects in our unbiased sample; there are no missing points or upper limits. For the FR Is, we have used the 7 with 178-MHz radio luminosity  $< 2 \times 10^{25} \text{ W Hz}^{-1} \text{ sr}^{-1}$  for which suitable data are available; there are 7 other FR Is for which there are no data but these have similar structures, sizes and radio luminosities to those used here and bias is unlikely. For the high- $z$  FR II radio sources, there are suitable data for 24 of the 28 possible radio galaxies and 9 of the 12 possible quasars. The spectrophotometric data for most of the objects were taken from refs 2, 11, 22, with additional data for 3C76.1 (ref. 23; an upper limit), 3C196 (ref. 24), 3C275.1 (ref. 25), the new identification for 3C326 (ref. 26), 3C334 (ref. 27), 3C338 (refs 8, 28). We measured a flux of  $\text{H}\alpha + [\text{N II}]$  of  $7.5 \times 10^{-18} \text{ W m}^{-2}$  for NGC6251 during the observing run described in ref. 29. For 3C31, 3C66B and 3C465, we used the data in ref. 30, calibrated against the stellar light of our NGC6251 spectrum (using an aperture correction), and obtained  $\text{H}\alpha + [\text{N II}]$  fluxes of 5.5, 11.0 and  $7.5 \times 10^{-18} \text{ W m}^{-2}$ , respectively. As these spectra imply a low ionization parameter we have assumed for these objects that  $L_{\text{H}\alpha + [\text{N II}]} \approx L_{[\text{O II}]}$ .  $L_{\text{NLR}}$  represents the total luminosity of all optical narrow lines and  $\text{Ly}\alpha$ , and is calculated<sup>7</sup> as  $3 \times (3 \times L_{[\text{O II}]} + 1.5 \times L_{[\text{O III}]})$ , where we have made use of the close relationships between the fluxes of low- and high-ionization lines with  $[\text{O II}]$  (3,727 Å) and  $[\text{O III}]$  (5,007 Å), respectively, and have adopted a median value for the ratio of total recombination-line radiation to total forbidden-line radiation. If only one oxygen line was measured we estimated the other, where possible, using measurements of other lines of similar ionization, otherwise we used the relation in ref. 29 at  $z < 0.5$  and  $L_{[\text{O III}]} = 4 \times L_{[\text{O II}]}$  at  $z > 0.5$  (ref. 22);  $L_{[\text{N II}]} ([\text{N II}] \text{ at } 6,548 \text{ and } 6,583 \text{ Å})$  was sometimes substituted for  $L_{[\text{O II}]}$ .

proportionality and its scatter will contrive to give quasars apparently higher  $L_{\text{NLR}}$  at constant  $Q$  and thus similar  $L_{\text{rad}}$ .

We now consider accretion rates, first in the low- $Q$  ( $Q \leq 10^{38}$  W) objects of Fig. 1. These often contain huge stored energies  $E$  ( $\sim 10^{53}$  J, equivalent to  $5 \times 10^5 M_{\odot}$ ) and must therefore contain central masses  $\geq 10^7 M_{\odot}$ , with corresponding Eddington luminosities  $L_{\text{Edd}} \geq 10^{38}$  W. For  $L_{\text{phot}}$  to exceed such values given the observed  $L_{\text{NLR}} \leq 10^{36}$  W, covering factors  $\kappa \leq 0.01$  are required, smaller than those observed in quasars (we assume equal power in photon flux per frequency decade); further, their low ionization parameters would require extreme gas densities in the narrow-line region (electron density  $n_e \gg 10^8 \text{ m}^{-3}$ ). Thus the accretion rate must be below the Eddington limit in low- $Q$  objects.

In high- $Q$  objects, where the energy stored in lobes is similar but  $L_{\text{phot}}$  much higher than in low- $Q$  objects, super-Eddington accretion cannot be similarly ruled out. Any change from sub- to super-Eddington accretion as a function of  $Q$  is achieved without a discontinuity in Fig. 1, however, as may be possible with the recent model of Bell<sup>12</sup>. Alternatively, in all objects with radio jets, the accretion may be sub-Eddington and the central masses correspondingly large. The fraction of the power from the nucleus that is channelled into jets supports this hypothesis. Figure 1 shows that this fraction is roughly the same for all objects and indeed even for  $\kappa \approx 0.01$ , the radiated and bulk kinetic power are roughly equal. Thus any process whereby accretion drives the jets directly would be implausibly efficient. (Both equipartition energy  $E$  and spectral age  $T$  have the same dependence on the volume-filling factor, which is the only factor that allows  $E$  to be an overestimate of the true lobe energy. Thus, as a decrease in  $\eta$  also increases  $Q$ , the values of  $Q$  we use are truly minimum ones, making an even better case for high efficiency.) We are led to a model in which the radiation arises from accretion, but the jet power is produced by a machine, which, to satisfy Fig. 1, must also control the accretion rate. A possible model involves jets that are driven by the extraction of rotational energy from a spinning black hole, ringed by an ion-supported torus<sup>13</sup>. Whatever the model, it must explain the two key features of the ratio of the outputs in radiated and bulk kinetic form seen in Fig. 1: (1) why it is at most of the order of unity; (2) why it is independent of the total power over four orders of magnitude. Both features may prove particularly challenging to models that exclude central massive black holes.

Finally, we emphasize that the objects we studied were known *a priori* to contain large-scale radio jets. Many objects have high narrow-line luminosities but negligible large-scale radio emission, such as the radio-quiet quasars. These do not obey the relation of Fig. 1. This implies that the formation of radio jets requires specific physical attributes independent of the accretion rate (such as that the central black hole be large and spinning), or that the bulk outflow is formed but then disrupted within the host galaxy. Either way, the radio-quiet and radio-loud objects are physically distinct.  $\square$

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## Evidence for a rotating helical filament in L1641, part of the Orion cloud complex

Y. Uchida\*, Y. Fukui†, Y. Minoshima†, A. Mizuno†, T. Iwata‡ & H. Takaba‡

\* Department of Astronomy, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

† Department of Astrophysics, Nagoya University, Chikusa-ku, Nagoya 464–01, Japan

‡ Communication Research Laboratory, Kashima-cho, Ibaraki 314, Japan

INTERSTELLAR cloud structures, typically 10–30 pc long and 3–5 pc wide, are often seen extending outwards from dense clouds that show marked enhancement of star formation within them. We have used the Nagoya 4-m radiotelescope to study one such 'streamer', L1641, a part of the giant molecular-cloud complex in Orion, lying south of the Kleinmann–Low (KL) nebula. Using the 110-GHz line of  $^{13}\text{CO}$  ( $J=1-0$ ), we have obtained intensity and velocity data, and find within the streamer a dense filament with a helical structure, spinning in the same sense as the gas in the Orion KL region. We propose a model for this structure in which the streamer, through the action of the interstellar magnetic field, acts as an angular-momentum drain on the Orion KL region, allowing it to collapse. In this model, the  $\sim 30$ -pc-long streamer is essential to the formation of the cloud, as well as the formation of stars within the dense cloud.

Figure 1 shows a contour map of the  $^{13}\text{CO}$  ( $J=1-0$ ) total intensity in the L1641 streamer obtained with the Nagoya 4-m telescope equipped with a low-noise superconductor-insulator-superconductor (SIS) detector<sup>1</sup>. This part of the streamer is characterized by the formation of low-mass protostars, showing molecular outflows<sup>2</sup>. We note a winding of the structure around the average direction of the cloud elongation as seen from the trace of local intensity peaks (the dots and curve in Fig. 1).

The upper panel of Fig. 2 shows the contours of gas having velocities  $\pm(0.6-1.5) \text{ km s}^{-1}$  relative to the average velocity along the  $X$  axis. We define this  $X$ -axis as the average direction of the winding structure ( $X$  increases with galactic longitude  $l$ ), and define an  $S$ -axis perpendicular to it ( $S$  increases with galactic latitude  $b$ ), as indicated. We use a method of analysis<sup>3</sup> in which the spinning of elongated object is isolated by first reducing the velocity field to  $v^*(X, S) = \{v(X, S) - v_{\text{mean}}(X)\}$ , where  $v_{\text{mean}}(X)$  is the doubly averaged velocity at each  $X$  along the elongated object, as described in the caption of Fig. 2, and then plot contour maps of  $v^*(X, S)$ . Using this method, a blob of gas with  $0 > v > v_{\text{mean}}(X)$  (or  $v_{\text{mean}}(X) > v > 0$ ), which is otherwise immersed in the blueshifted (or redshifted) gas and not thus obvious unless many velocity slices are examined and then resynthesized, becomes readily visible as a part redshifted (or blueshifted) relative to  $v_{\text{mean}}(X)$ .

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The gas that is blueshifted relative to  $v_{\text{mean}}(X)$  is always located above the axis as regularly spaced patches, whereas the gas with a relative redshift is seen below the axis, and the blueshifted and redshifted patches alternate. The typical position-velocity diagrams shown in the lower panels of Fig. 2 indicate that the velocity gradient is  $\approx 1 \text{ km s}^{-1} \text{ pc}^{-1}$ . The shape of the structure and the morphology of the velocity distribution strongly suggest a helical structure.

There are several possible empirical models for the observed velocity field. (1) A flow of a few kilometres per second in the direction of increasing  $X$ , along a (fixed) left-handed (seen from the direction of Ori KL) helical path towards the end of the streamer—that is, outflow, if the Ori KL region is the source. (2) A flow of a few kilometres per second in the direction of decreasing  $X$  along a (fixed) right-handed (seen from the direction of Ori KL) helical path, coming into the helical filament from the end of the streamer (inflow towards the direction of Ori KL). (3) Rotation of a right-handed (seen from the direction of Ori KL) helical pattern in a left-handed circular direction. The flow can be either towards increasing or decreasing  $X$ , but in the former case the azimuthal component of the flow velocity  $v_\phi$  should be smaller than the rotation velocity of the helical pattern around its axis.

Using circumstantial evidence, we can select the most likely of these possibilities. If we attribute the helical structure to the structure of the magnetic field, then it is natural to think that the winding direction of the helix is the same as that of the large-scale magnetic-field configuration surrounding our helix. Bally and Uchida have noted from the polarization of the background star light<sup>4</sup> and measurements of the Zeeman effect<sup>5</sup> that the magnetic field surrounding L1641 has a helical structure winding around the streamer in a right-handed (seen from the direction of Ori KL) helix, as shown in Fig. 10 of ref. 6. With this restriction, case (1) above can be dropped. In cases (2) and (3), the inflow does not seem very natural. Non-magnetic activities such as radiative or stellar wind pressure cannot make the gas flow into the special channels we observe, and there is no trace of magnetic activity to the left. Thus, we prefer the case in which a right-handed helical structure (with the same sense as the winding structure in the large-scale magnetic field around L1641 inferred by Bally and Uchida) is rotating in the left-

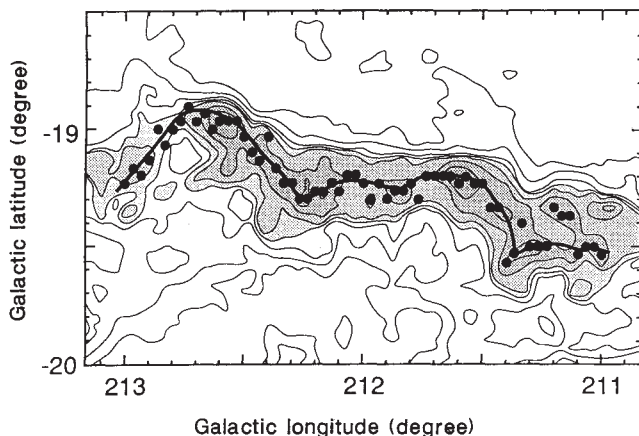


FIG. 1 Contour map of the  $^{13}\text{CO } J=1-0$  total intensity for the winding structure in L1641. Dots indicate local peaks of intensity for each galactic longitude  $l$ , and the solid line is a guide to the eye. The observations were made from January to February 1990 with the 4-m radiotelescope at Nagoya University equipped with a 4-K-superconducting mixer receiver having a receiver temperature of 25 K in double side band<sup>1</sup>. The beam size of the telescope was 2.7 arcmin at 110 GHz, and the data were sampled every 2 arcmin with typical r.m.s. noise fluctuations of 0.5 K at  $0.1 \text{ km s}^{-1}$  velocity resolution. The contours are 2, 4, 6, 7, 8, 9, 10, 11, 12 and  $13 \text{ K km s}^{-1}$ . The areas with intensities  $> 7 \text{ K km s}^{-1}$  are shaded.

handed direction. The gas flow is toward increasing  $X$  (outflow in terms of the Ori KL region) with the  $\phi$  component (right-handed circular) somewhat smaller than the left-handed circular rotation velocity of the helical pattern itself, giving net motion as observed. It is remarkable that this situation is consistent with the case in which angular momentum is being carried towards the left from the source on the right twisting the flux tube.

Figure 3 shows the result of a fit using, for simplicity, a simple uniform helix model. In the model, the spatial and velocity deviations of the periodic component are expressed as  $S = a \cos \theta$  and  $\Delta v = (v_{\text{flow}} \cos \alpha + v_{\text{rot}}) \cos \theta \sin i$ , where the parameter  $\theta$  is related to  $X$  by  $X = a[(\sin \theta - 1) \cos i + (\theta - \pi/2) \tan \alpha \sin i]$ . First the parameters of the helix,  $a$ ,  $\alpha$  and  $i$ , were determined to be 1 pc ( $\approx 7''$ ),  $53^\circ$  and  $110^\circ$ , respectively, by a least-squares fit to the spatial pattern in the upper panel. Then  $(v_{\text{flow}} \cos \alpha + v_{\text{rot}})$  was determined to be  $-0.5 \text{ km s}^{-1}$  from the velocity distribution in the lower panel for this set of  $a$ ,  $\alpha$  and  $i$ . Both fits are good, as shown by solid lines in each panel. Without additional constraints,  $v_{\text{flow}}$  and  $v_{\text{rot}}$  are not uniquely determined. One possible solution is  $v_{\text{rot}} \approx -1 \text{ km s}^{-1}$ ,  $v_{\text{flow}} \approx 1 \text{ km s}^{-1}$  and  $i = 110^\circ$ , but other combinations, such as  $v_{\text{rot}} \approx -2 \text{ km s}^{-1}$ ,  $v_{\text{flow}} \approx 3 \text{ km s}^{-1}$  and so on, also fulfil

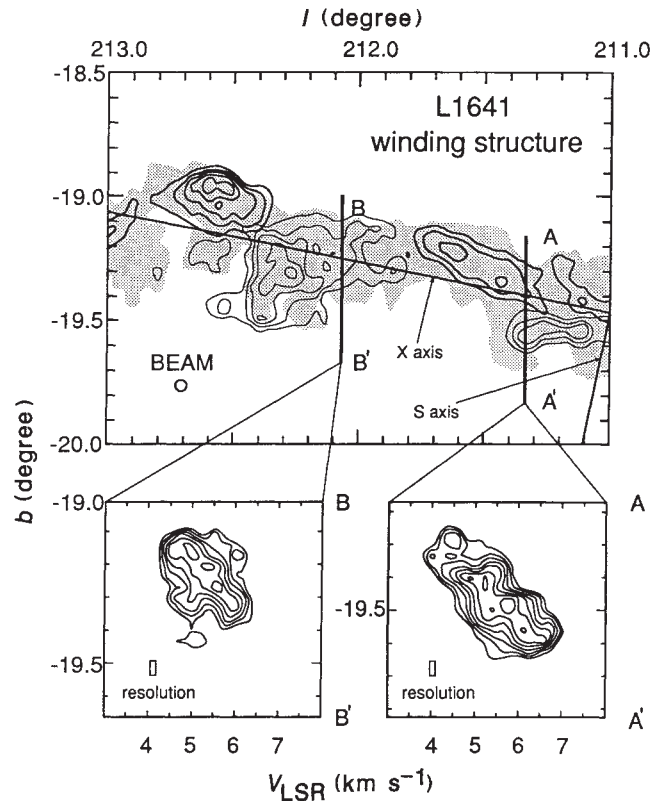


FIG. 2 Velocity distribution in the winding structure in L1641. We use an analysis similar to the one we used for the  $\rho$  Ophiuchi streamer<sup>3</sup>. The analysis is as follows. At a given galactic longitude we averaged  $^{13}\text{CO}$  profiles in the shaded area ( $^{13}\text{CO}$  total intensity  $\geq 8 \text{ K km s}^{-1}$ ). Averaged  $^{13}\text{CO}$  profiles,  $T_{\text{av}}(l; v_{1,\text{sr}})$ , were then used to calculate  $v_{\text{av}}(l)$  by equating it to  $\int T_{\text{av}}(l; v_{1,\text{sr}}) v_{1,\text{sr}} dv_{1,\text{sr}} / \int T_{\text{av}}(l; v_{1,\text{sr}}) dv_{1,\text{sr}}$ .  $v_{\text{av}}(l)$  shows a decrease of  $\sim 1 \text{ km s}^{-1}$  from  $l = 211$  to  $213$ , exhibiting a sinusoidal pattern of amplitude  $\sim 1 \text{ km s}^{-1}$ . To remove the large-scale trend, we approximated  $v_{\text{av}}(l)$  with a straight line  $v_{\text{mean}}(l)$  by a least-squares fit. The thick solid contours of the upper panel correspond to velocities of  $-1.5$  to  $-0.6 \text{ km s}^{-1}$  relative to  $v_{\text{mean}}(l)$ , and the thin solid contours show velocities of  $0.6$  to  $1.5 \text{ km s}^{-1}$  relative to  $v_{\text{mean}}(l)$ . Contours are plotted every  $0.5 \text{ K km s}^{-1}$  from the lowest one at  $2.5 \text{ K km s}^{-1}$ . In the lower panels, position-velocity diagrams at  $l = 211.33^\circ$  and  $212.07^\circ$  are shown as examples. Contours are plotted every  $0.3 \text{ K}$  from the lowest one of  $2.7 \text{ K}$ ; velocity resolution is  $0.1 \text{ km s}^{-1}$ .

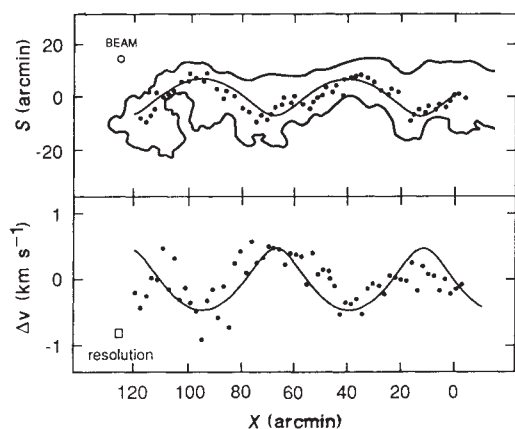


FIG. 3 Results of the fit to a uniform helical model. Rotational velocity of the helical pattern,  $v_{\text{rot}}$ , and the velocity of the gas flowing along it,  $v_{\text{flow}}$ , are taken to be positive for right-handed rotation seen from Ori KL, and for outflow from the Ori KL region, respectively.  $(X, S) = (0, 0)$  corresponds to  $(l, b) = (211.0^\circ, -19.45^\circ)$  and  $(X, S) = (120', 0)$  to  $(l, b) = (212.93^\circ, -19.07^\circ)$ . We considered the relative velocity  $\Delta v(X) \approx \Delta v(l)$ , although the  $(X, S)$  coordinate is tilted somewhat from the  $(l, b)$  coordinate. This error is probably not important because it does not exceed  $2'$ , the grid spacing, in the area analysed. The helix, right handed toward the direction of increasing  $X$ , is assumed to have a constant pitch angle  $\alpha$  and a radius  $a$ , and its axis directed toward the positive  $X$  is at an angle  $i$  with the line of sight. In the model, we considered the filament's width to be infinitesimal and did not take into account the internal structure of the helix. The dots in the upper panel show peak positions of the  $^{13}\text{CO}$  total intensity for each  $X$ , and the dots in the lower panel show  $\Delta v \approx v_{\text{av}}(l) - v_{\text{mean}}(l)$ . The curves denote values calculated for the uniform-helix model.

( $v_{\text{flow}} \cos \alpha + v_{\text{rot}}$ )  $\approx -0.5 \text{ km s}^{-1}$ . The axis of the helix is somewhat tilted toward us with the increasing  $l$ . These explain the distribution pattern of the line-of-sight velocity and the fact that bending is sharper in the lower half of the helix than in the upper half. This situation, gas flow along a right-handed helix which is spinning in the left-handed circular direction, giving a net left-handed helical motion in the rest frame, is consistent with magnetic transport of angular momentum from the 'spinner' (probably the mass and angular momentum of the Ori KL region as a whole) to the streamer<sup>3,7</sup>.

The magnetic field strength may be estimated from the flux-conserving contraction of the mass together with the background field of a few microgauss into that of the steamer, giving  $B = 100 \mu\text{G}$ . This is consistent with the pattern velocity, which is related to the nonlinear torsional Alfvén velocity in the filament,  $V_A \approx 5\text{--}15 \text{ km s}^{-1}$ , by which the helical pattern has propagated to a distance of 10–30 pc in the lifetime of Ori KL and its related region, the 'spinner' in our picture.

We envisage the situation as follows (Y.U., K. Shibata and R. Rosner, manuscript in preparation): we assume a contraction of a massive cloud taking place in a medium having a large-scale magnetic field. The magnetic field is deformed into an 'hourglass' shape as the massive condensed cloud is formed at the centre (the neck of the 'hourglass'). We will discuss elsewhere the explanation of the observed asymmetry in terms of the same effect propagating from the formation of massive clouds on the northern side of Ori KL, where we see a few clusters of OB stars that were formed earlier than that in the Ori KL region.

The toroidal component of the magnetic field is continuously produced through differential rotation inside the dense cloud. When the toroidal component relaxes along the hourglass-shaped poloidal field, it wraps (weakly pinches) the poloidal field into a cylindrical rod-like shape. The confinement of the rotating gas is due to the magnetic field and self-gravity. We identify this 'wrapped up' structure, having a helical field in and around it, with the streamer. We also identify the winding filament in it with part of a wound-up flux tube in the streamer produced by the above process. This flux tube is made apparent

by the flow from the 'sequential star-formation process'<sup>9,10</sup> occurring along it.

If this model is correct, the production of the spinning streamer loaded with mass exerts a magnetic braking on the massive cloud. This will allow the massive cloud to shrink to a denser cloud in which star formation may take place more efficiently<sup>11</sup>, and may explain the high rate of star formation within it.  $\square$

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## Origin of sulphur in Canadian Arctic haze from isotope measurements

Jerome O. Nriagu\*, Robert D. Coker\* & Len A. Barrie†

\* National Water Research Institute, Box 5050, Burlington, Ontario, Canada L7R 4A6

† Atmospheric Environment Service, 4905 Dufferin Street, Downsview, Ontario, Canada M3H 5T4

SINCE the mid-1950s, there has been a marked increase in levels of air pollution in the Arctic region<sup>1–3</sup>. This is apparent from the pervasive haze that has been detected over large areas of the Northern Hemisphere<sup>3–6</sup>, which can cover up to 9% of the Earth's surface. The haze is most pronounced during January to April, and is characterized by a reduction in visibility and anomalously high levels of organic and inorganic compounds of the type found in polluted environments<sup>3,6,7</sup>. Most of the present knowledge about the origin of the chemical constituents of the haze comes from studies of elemental ratios and trajectory analyses<sup>2,4,6,7</sup>. Here we report the results of an analysis of the isotopic composition of sulphur in the Arctic haze. We find that most of the sulphur in the haze comes from Europe rather than from more local anthropogenic or biogenic sources, indicating that this form of air pollution becomes distributed globally.

The samples used here are from the archived aerosol collection at the Atmospheric Environment Service (Downsview, Ontario). Alert (82.5° N, 62.3° W), on Ellesmere Island, and Mould Bay (76.2° N, 119.3° W), on Prince Patrick Island, are stations in the Canadian Arctic aerosol sampling network. Weekly aerosol samples were collected from each station by drawing 16,000 m<sup>3</sup> of air through 25 × 20 cm Whatman 41 filters. Field blank filters were collected every four weeks and analysed in the same way as the samples. To exclude the influence of local sources, the sampler at Mould Bay was located about 1.5 km upwind of the camp site, and at Alert it was placed on a 200-m plateau 6 km from the camp. Details of the sampling stations and the air sampling strategy are given in refs 8 and 9.

A section of each filter paper was leached with distilled water and the sulphate concentration in the leachate measured using an ion chromatograph. We believe that the reproducibility in the determination of the sulphate aerosol concentration in the air is  $\pm 13\%$  of the reported value<sup>9</sup>. Another section of the filter

paper was extracted ultrasonically in distilled water. The sulphate in the extract was recovered as  $\text{BaSO}_4$  and then converted to  $\text{SO}_2$  by direct thermal decomposition<sup>10,11</sup>. Because of the low ambient  $\text{SO}_4$  concentrations in summer months, we combined samples collected during several weeks to obtain enough sulphur for the isotope measurements. The isotope ratio of the  $\text{SO}_2$  was measured using a VG Micromass 602C mass spectrometer. The reproducibility of the  $\delta^{34}\text{S}$  measurements is  $\pm 0.3\%$ , based on replicate analyses of the filter papers.

The ground-level concentrations of sulphate at Alert are highest in winter (January to April) and lowest in summer, with the difference between the winter and summer concentrations being over tenfold (Fig. 1). Similar seasonal variations have also been reported in many other regions of the Arctic, with the winter concentrations sometimes approaching the levels found in rural and urban areas<sup>6,12,13</sup>. There are significant differences in the amplitude of the peaks, which have been attributed to peculiar weather conditions during each winter<sup>3</sup>. There are a number of detailed discussions of the spatial and temporal variations in the chemical composition of Arctic aerosols, which also show that sulphate is a major component (up to 30%) of the aerosols<sup>2-9</sup>.

The  $\delta^{34}\text{S}$  data for the weekly aerosol samples at Alert also show pronounced seasonal differences, with the sulphur being heavier in summer than in winter (Fig. 2). The  $\delta^{34}\text{S}$  for the haze is remarkably constant from year to year, the average values (in

%, with  $1\sigma$  errors and the number of samples,  $n$ ) being  $+5.7 \pm 0.67$  ( $n=20$ ) during the winter of 1983/84,  $+5.6 \pm 0.62$  ( $n=20$ ) for 1984/85 and  $+5.9 \pm 0.54$  ( $n=19$ ) during 1985/86. The isotopic composition of sulphur aerosols at Mould Bay also shows a very similar seasonal behaviour (Fig. 2) and the average  $\delta^{34}\text{S}$  at this station during the winter of 1983/84 is  $+5.9 \pm 0.45$  ( $n=9$ ), almost identical to that at Alert. The uniformity in chemical characteristics of the haze throughout the Arctic region is already well documented<sup>2,14-16</sup>. The small inter-winter differences in the weekly pattern of the  $\delta^{34}\text{S}$  values (Fig. 2) can be attributed to differences in the weather conditions and meridional transport processes<sup>17</sup>.

The key features of the isotopic composition of Arctic sulphur are quite different from those of airborne sulphur in southern Canada. For example,  $\delta^{34}\text{S}$  values for the haze are higher than those of the atmospheric sulphur in urban and rural areas of eastern and central North America, which are generally 0–5% (refs 10, 18–21). More important, the nature of the seasonal variations is very different. In rural and remote areas of southern Canada, the  $\delta^{34}\text{S}$  values of atmospheric samples peak in winter and are lowest during the warm summer months<sup>10,18</sup>. By contrast, the heaviest sulphur is observed in the Arctic during the summer months (Fig. 2). These differences suggest that the atmospheric sulphur in the Arctic has a different origin from that in southern Canada.

In southern Canada, most of the airborne sulphur during the winter period is derived from space heating and industrial sources, and the  $\delta^{34}\text{S}$  generally reflects the regional source pattern. In summer, the large emission of lighter biogenic sulphur from soils, vegetation, marshes and wetlands results in the lowering of the  $\delta^{34}\text{S}$  values of the airborne sulphur<sup>10,18,21-22</sup>. This is because the  $\delta^{34}\text{S}$  of terrestrial ecosystems in southern Canada typically is  $<5\%$  and the biogenic sulphur is depleted in  $^{32}\text{S}$  relative to the parent material<sup>18</sup>. The difference in the isotope signature indicates that these dominant natural and anthropogenic sources in southern Canada make only a minor contribution to the Arctic sulphur pool. Indeed, meteorological and tracer studies as well as transport models using emissions inventories suggest that only a small fraction ( $<10\%$ ) of the sulphur flux to the Arctic comes from North America<sup>23-25</sup>.

The isotope data can be related to differences in the sources of sulphur in the Arctic during the winter and summer months. Sulphur in Arctic air can be derived from anthropogenic sources, sea-salt aerosols, the oxidation of biogenic sulphur compounds and, to a lesser extent, from entrained dust particles<sup>26-29</sup>. Measurements of air pollutants as well as chemical/transport models generally suggest that up to 80% of the sulphur in the Arctic haze is derived from anthropogenic sources in Europe<sup>3,7,26</sup>. Published data on the isotopic composition of the atmospheric sulphur from sources in the Soviet Union and eastern Europe are extremely sparse and range from  $+1.0$  to  $>+21\%$  (ref. 30); the available information does not include anything on the  $\delta^{34}\text{S}$  of the Arctic sulphur pool. The mean  $\delta^{34}\text{S}$  for rainfall sulphate in the Soviet Union,  $+5.9\%$  ( $n=49$ , see ref. 31), falls within the range of the isotopic composition of the haze.

In an effort to verify the European origin of the sulphur in the Canadian Arctic, we determined the  $\delta^{34}\text{S}$  of aerosols collected at Ny Alesund (Norway) throughout the month of January 1988. The average  $\delta^{34}\text{S}$  value of  $+6.4 \pm 0.82\%$  ( $n=13$ ) for the Norwegian samples (see Table 1) is comparable to the data for Alert and Mould Bay and points to a common origin. These results are in agreement with those obtained from measurements of stable lead isotopes made by Sturges and Barrie<sup>16</sup> using aliquots of the same samples collected between November 1983 and May 1984. They found that the mean  $^{206}\text{Pb}/^{207}\text{Pb}$  ratios of  $1.160 \pm 0.010$  at Alert and  $1.161 \pm 0.006$  at Mould Bay were very close to that of aerosol samples at Spitsbergen (Norwegian Arctic) during air flow from the Soviet Union and also to the mean isotope ratio of  $1.158 \pm 0.014$  for non-ferrous ore deposits of the Soviet Union. We have also analysed some of the daily

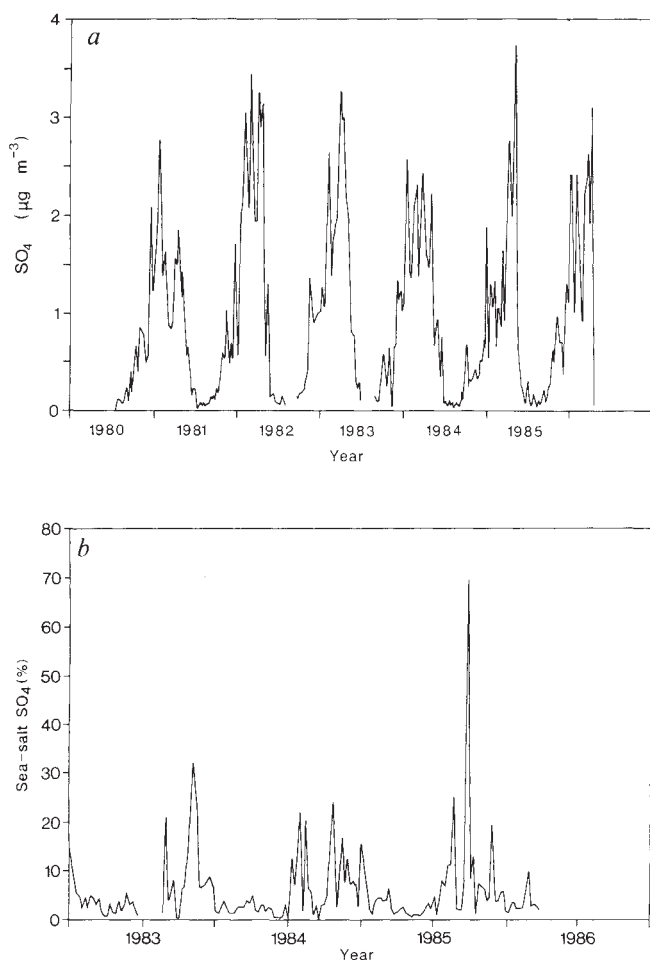


FIG. 1 *a*, Average weekly, ground-level concentrations of  $\text{SO}_4$  at Alert. *b*, The percentage of sea-salt sulphate in the aerosol was calculated using 0.25 as the  $\text{SO}_4$ :Na ratio in sea water.



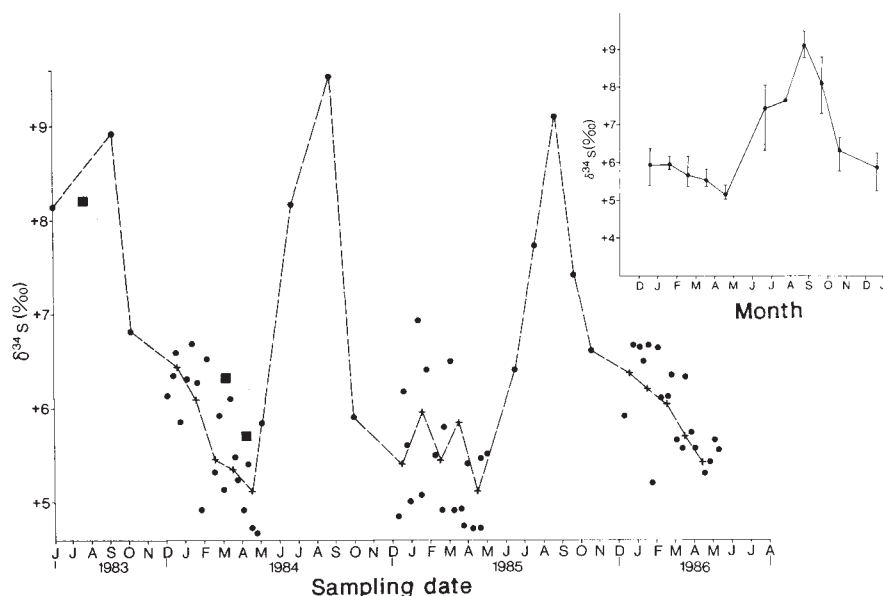


FIG. 2 Isotopic composition of sulphur in aerosols at Alert (circles) and Mould Bay (squares). The monthly average  $\delta^{34}\text{S}$ , with ranges, are shown as an insert.

aerosol samples collected during the spring of 1988 at Aspverten, ~100 km south of Stockholm, Sweden. We selected the samples according to origin using air-parcel back trajectories. The  $\delta^{34}\text{S}$  values for the sulphur from western European, USSR and eastern European sectors are fairly similar (Table 1). The  $\delta^{34}\text{S}$  values fall within the range observed in the Canadian Arctic although the average value for the Swedish samples of  $+5.0 \pm 1.0\%$  is slightly lower than those of the haze (Fig. 2). The discrepancy may be attributed to the additional inputs of heavier

sulphur from marine sources (see below) to the polluted air masses as they travel to North America<sup>27-29</sup>.

In contrast to the winter time, when industrial emissions account for most of the airborne sulphur, the contributions from sea-salt sprays and biogenic sources are of paramount importance during the summer<sup>6,16,17,27-29</sup>. Sea-salt aerosols typically account for 10–30% of the atmospheric sulphur burden during the summer (Fig. 1). The  $\delta^{34}\text{S}$  value for sea-salt aerosols can be assumed to be identical to that of sea water, or ~20‰ (ref. 31). The average flux of dimethyl sulphide (DMS) from the oceans above the 60° N in summer ( $280 \mu\text{g m}^{-2} \text{yr}^{-1}$ ) is about ten times higher than in winter months<sup>32</sup> ( $31 \mu\text{g m}^{-2} \text{yr}^{-1}$ ). Measurements of methanesulphonic acid in the Arctic air and analysis of the principal components in the aerosols suggest that a large fraction (>50%) of the non-sea-salt sulphur may be produced from the oxidation of the DMS derived biogenically from the Arctic Ocean, marine sources in the lower latitudes and possibly from terrestrial ecosystems near the Arctic circle<sup>7,29</sup>. The isotopic composition of marine DMS is unknown but is believed to be <10‰ lighter than that of sea water sulphur<sup>21</sup>. If sea-salt spray and marine biogenic sources were responsible for all of the airborne sulphur during the Arctic summer, the expected  $\delta^{34}\text{S}$  value would fall in the range 10–20‰; rainfalls in the open ocean generally fall in this range<sup>31</sup>. Our data in Fig. 2, however, show summer time  $\delta^{34}\text{S}$  values of 7–10‰, implying further inputs of lighter sulphur, believed to be of anthropogenic origin. Local soils represent a very minor source of aerosols at the sampling stations<sup>3,8</sup>.

The isotope data are thus consistent with previous studies which show that the sulphur in Arctic haze comes primarily from anthropogenic sources in Eurasia. In summer, however, a large fraction of the atmospheric sulphur burden can be derived as sea-salt sprays and from oxidation of DMS released from marine ecosystems. Quantification of the relative contributions of sulphur from the various sources, however, is impossible on the basis of the current isotope measurements. □

TABLE 1 Isotopic composition of sulphur aerosols

| Sampling date<br>(1988) | Air source* | $\delta^{34}\text{S}^\dagger$ |
|-------------------------|-------------|-------------------------------|
| Ny Alesund, Norway      |             |                               |
| 1–4 January             | Unknown     | +6.9                          |
| 4–6 January             | Unknown     | +7.4                          |
| 6–8 January             | Unknown     | +6.6                          |
| 8–11 January            | Unknown     | +5.1                          |
| 11–13 January           | Unknown     | +5.5                          |
| 13–15 January           | Unknown     | +6.4                          |
| 15–18 January           | Unknown     | +6.6                          |
| 18–21 January           | Unknown     | +7.7                          |
| 21–22 January           | Unknown     | +7.1                          |
| 22–25 January           | Unknown     | +6.5                          |
| 25–27 January           | Unknown     | +6.4                          |
| 27–29 January           | Unknown     | +5.4                          |
| 29 January – 1 February | Unknown     | +5.4                          |
| Aspverten, Sweden       |             |                               |
| 10 February             | W. Europe   | +7.6                          |
| 11 February             | W. Europe   | +6.3                          |
| 14 February             | W. Europe   | +5.4                          |
| 3 May                   | W. Europe   | +4.6                          |
| 4 May                   | W. Europe   | +4.4                          |
| 23 February             | USSR        | +5.0                          |
| 24 February             | USSR        | +4.2                          |
| 25 February             | USSR        | +4.4                          |
| 26 February             | USSR        | +4.7                          |
| 22 March                | USSR        | +3.8                          |
| 23 March                | USSR        | +4.4                          |
| 15 February             | E. Europe   | +4.4                          |
| 16 February             | E. Europe   | +6.1                          |
| 16 April                | E. Europe   | +5.1                          |
| 2 May                   | E. Europe   | +4.4                          |

\* Determined using air-parcel trajectories.

$\dagger \delta^{34}\text{S}(\text{‰}) = 1,000 \times [({}^{34}\text{S}/{}^{32}\text{S})_{\text{sample}} / ({}^{34}\text{S}/{}^{32}\text{S})_{\text{standard}} - 1]$ , where the standard was Canyon Diablo troilite.

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## Air-sea gas exchange in rough and stormy seas measured by a dual-tracer technique

Andrew J. Watson\*, Robert C. Upstill-Goddard\*† & Peter S. Liss†

\* Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK

† School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, UK

THE transfer of dissolved atmospheric gases across the air/sea interface is a strong function of wind speed and state of the sea surface. The form of the dependence on wind velocities is needed to calculate oceanic sources and sinks of climatically significant gases, particularly carbon dioxide<sup>1</sup> and dimethylsulphide<sup>2,3</sup>. Previous measurements at sea have used techniques that reveal this dependence only when averaged over weeks or months<sup>4,5</sup>, making it impossible to study the effects of high winds, which are generally episodic. Here we report first results from the application of a tracer technique which enables accurate measurements to be made over periods of 24–72 hours, including the first determination of gas exchange in storm conditions. Our results support the wind-speed dependence suggested by Liss and Merlivat<sup>6</sup> on the basis of lake and wind-tunnel experiments, and are consistent with most measurements made by the radon-deficit technique. They suggest slower rates for CO<sub>2</sub> exchange than those obtained by <sup>14</sup>C budgeting, and favour recent lower estimates<sup>1,7</sup> of the global oceanic sink for anthropogenic CO<sub>2</sub>.

Our technique uses the simultaneous release into the water column of small quantities of the two inert, non-toxic gaseous tracers sulphur hexafluoride (SF<sub>6</sub>) and <sup>3</sup>He. The tracers transfer to the atmosphere at different rates, so that the ratio of their concentrations changes with time. It can be shown that, in a region of water that is well stirred from the surface to the sea bed, the ratio changes according to

$$(1/R) dR/dt = -(K_{He} - K_{SF_6})/H \quad (1)$$

where  $K_{He}$  and  $K_{SF_6}$  are the transfer velocities of the gases across the air/sea interface and  $H$  is the water depth.  $R$  is the ratio of the tracer concentrations, taking into account any background that existed in equilibrium with the atmosphere before the release— $R = \{[{}^3\text{He}] - [{}^3\text{He}_{eq}]\} / \{[\text{SF}_6] - [\text{SF}_{6,eq}]\}$ . Ideally, one of the tracers should be non-volatile (a transfer velocity of zero), in which case the rate of change of  $R$  would directly give  $K$  for the volatile tracer. At present no environmentally acceptable and operationally suitable non-volatile tracer is available, and

the quantity actually deduced from measurements of  $R$  is the difference between the transfer velocities of SF<sub>6</sub> and <sup>3</sup>He. To obtain the absolute transfer velocities we require an additional equation relating  $K$  for the two gases under similar conditions. Models of gas exchange<sup>8</sup> suggest that the variation of  $K$  with the physical properties of the gases (and also with temperature) can be described by a power-law dependence on the Schmidt number  $Sc$ , so that for two gases

$$K_1/K_2 = (Sc_2/Sc_1)^n \quad (2)$$

where  $Sc$  is the kinematic viscosity of water divided by the molecular diffusivity of the gas in water at a given temperature. Both theory<sup>8,9</sup> and experiment<sup>9,10</sup> suggest that in the 'rough surface' regime normally applicable at sea,  $n \approx 0.5$ . We investigated this relationship for the particular case of the SF<sub>6</sub>–<sup>3</sup>He tracer pair by releasing them into a small freshwater lake and using a budgeting technique<sup>11</sup> to obtain absolute values for their transfer velocities. Table 1 shows the results obtained at two different wind speeds. The mean value for  $n$  in these experiments was 0.51, confirming the  $Sc^{-0.5}$  dependence. We adopt the value of 0.51 in the following.

We carried out two experiments at sea as part of the NERC North Sea Project, during March and October 1989. A permanently well mixed, flat-bottomed and shallow ( $30 \pm 2$  m) region of the southern North Sea off the Dutch coast was selected for study ( $52^\circ 15' - 52^\circ 30' \text{ N}$ ,  $3^\circ 20' - 3^\circ 40' \text{ E}$ ). We dissolved  $\sim 40$  g of SF<sub>6</sub> and 0.15 g of <sup>3</sup>He in one tonne of sea water in a tank

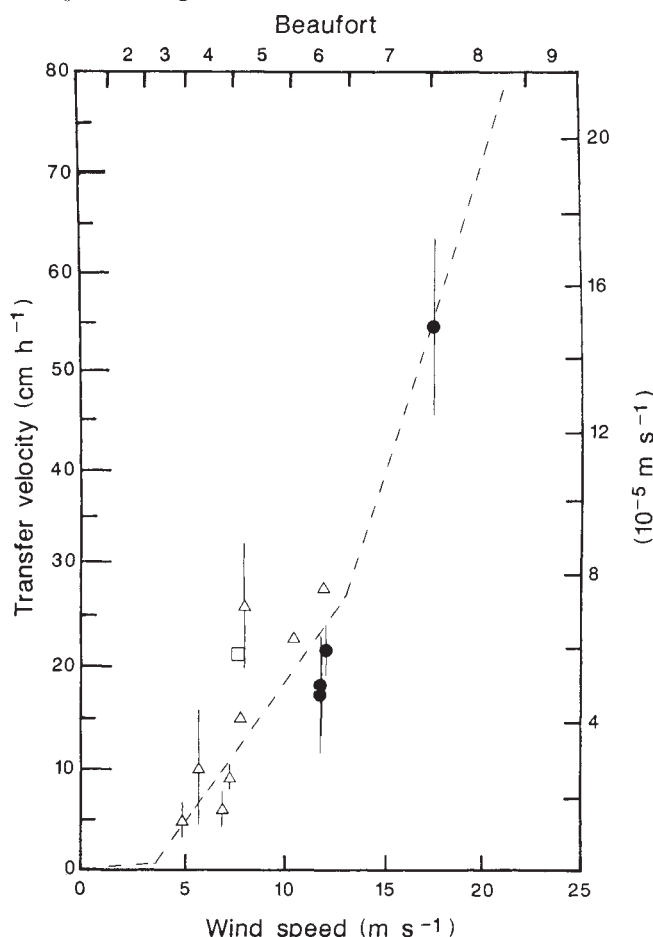


FIG. 1 CO<sub>2</sub> air-sea transfer velocities at 20 °C as a function of wind speed at a height of 10 m and the corresponding Beaufort Scale number. The results from this study are shown as filled circles. The open triangles are ocean radon averages<sup>4,5</sup> (those with no error bars shown are nevertheless averages of data sets showing very considerable scatter). □, Global mean 'bomb' <sup>14</sup>C CO<sub>2</sub> transfer velocity<sup>16</sup>. The dashed line represents the theoretical Liss and Merlivat relationship<sup>6</sup>, determined using data from lake and wind tunnel experiments.

TABLE 1 Determination of  $n$  in equation (2)

| Date             | Period $\Delta T$<br>(h) | Wind speed<br>(m s <sup>-1</sup> ) | $K(\text{SF}_6)$<br>(cm h <sup>-1</sup> ) | $K(^3\text{He})$<br>(cm h <sup>-1</sup> ) | $\text{Sc}(\text{SF}_6)^*$ | $\text{Sc}(^3\text{He})^\dagger$ | $n^\ddagger$ |
|------------------|--------------------------|------------------------------------|-------------------------------------------|-------------------------------------------|----------------------------|----------------------------------|--------------|
| 23 November 1988 | 46.5                     | 3.8                                | 2.27 ± 0.85                               | 7.10 ± 0.30                               | 2,200                      | 230                              | 0.505        |
| 28 November 1988 | 111.9                    | 5.8                                | 3.23 ± 0.36                               | 10.33                                     | 2,200                      | 230                              | 0.515        |

From a gas-budgeting study in a small lake (mean depth, 5.7 m; fetch, ~600 m).

\* Estimated from the equation of Hayduk and Laudie<sup>15</sup> for diffusion coefficient.

† Estimated from the data of Jähne *et al.*<sup>16</sup> for  $^4\text{He}$ , corrected to  $^3\text{He}$  by multiplying by  $\sqrt{3/4}$  and interpolated for temperature.

‡ Calculated as  $n = \ln(K_{\text{He}}/K_{\text{SF}_6}) / \ln(\text{Sc}_{\text{SF}_6}/\text{Sc}_{\text{He}})$ .

having negligible head space. The tank contents were then released through a hose at a depth of 10 m, by displacing the tracer-rich tank water with new water so that the contents were not exposed to the atmosphere. This procedure ensured that when the tracers were first released they were in a constant ratio to each other. Strict precautions were necessary to avoid cross-contamination of the sampling and measurement apparatus aboard the ship with the concentrated tracers: in the first experiment the release was made from an auxiliary vessel, and in the second the tank was charged with the tracers and sealed before the cruise.

After release the 'tagged' patch of water was mapped at intervals for a period of up to 10 days. Our apparatus allowed the continuous, near real-time measurement of  $\text{SF}_6$  in the surface water using electron-capture gas chromatography (EC-GC) so that we could map the dispersal and advection of the patch. This spread rapidly under the influence of the tide into an area of about  $5 \times 10$  km, but advected only a matter of a few kilometres during the experiments. To determine the ratio of the two tracers, we first surveyed the patch for  $\text{SF}_6$  to find its centre, at which point multiple samples were normally taken through the water column using standard Niskin bottles.  $\text{SF}_6$  analyses were carried out immediately on board whereas the  $^3\text{He}$  samples were returned to land for mass spectrometric analysis. The analytical precision of the ratio determinations was ~2%. (The individual analytical precisions of both  $\text{SF}_6$  and  $^3\text{He}$  measurements were of the order of 1%.) Variations in the ratio with depth were typically  $\leq 5\%$ . Details of the experimental procedure will be published elsewhere.

The experiments resulted in four independent estimates of gas transfer velocity (Table 2) obtained by measuring the change in  $R$  over intervals of 25 to 58 hours. (Several further determinations were attempted on the second cruise, but we have excluded these from the data because of problems with the onboard  $\text{SF}_6$  analysis.) Of the successful measurements, three are at almost the same average wind speed, but the fourth was made during a severe storm, when wind speeds averaged  $17.5 \text{ m s}^{-1}$  and gusted up to  $25 \text{ m s}^{-1}$ . (The vector wind was recorded continuously from a transducer mounted on a standoff from the ship's mast, and corrected for the ship's motion by subtraction of its vector

velocity, also continuously recorded.) Weather conditions during the storm made sampling from depth impossible, and this data point was therefore averaged from three surface determinations taken 52, 59 and 64 h after the initial measurement. These ratios showed more scatter than those obtained from depth profiles, but because the change in the ratio was comparatively large during the storm, the fractional precision of the determination of transfer velocity is similar to that for the results at lower wind speed.

Figure 1 shows our measurements as a function of wind speed, compared to other theoretical and experimental studies of gas exchange at sea. All data have been scaled to  $\text{Sc} = 600$ , corresponding to  $\text{CO}_2$  at  $20^\circ\text{C}$ , using equation (2). A small correction has been applied to adjust the wind speeds to a height of 10 m. Our method yields values of  $K$  that are somewhat lower than previous estimates derived from radon-deficit measurements at intermediate wind speeds<sup>4</sup>. But this is to be expected because, as such measurements are normally averaged over periods of weeks or months and may include a wide range of wind conditions, they will tend to lie above the curve of  $K$  plotted against wind speed which is markedly nonlinear (Fig. 1). There are no previous measurements with which to compare the value obtained during the storm, but it agrees well with the prediction of Liss and Merlivat<sup>6</sup>, and confirms wind-tunnel studies<sup>12</sup>, which suggest that the slope of the relationship between transfer velocity and wind speed should steepen as the surface begins to break up with whitecaps.

Our lake determinations of the Schmidt number dependence (Table 1) were made at wind speeds where whitecaps were absent. Once bubbles from breaking waves form, gas transfer is augmented by a process for which  $n$  in equation (2) may not be 0.5. Recent laboratory investigations of the Schmidt number dependence for the  $\text{SF}_6$ - $^4\text{He}$  pair suggest that  $n$  may decrease from 0.5 as the breaking wave regime is entered (R. Wanninkhof and W. Asher, personal communication). If this is found to be true in the sea, it will be necessary to revise upwards our estimate of the transfer velocity under storm conditions.

One consequence of  $\text{CO}_2$ -induced climate change may be to affect global wind speeds and the frequency of storms, in which case the steep increase in transfer velocities with wind will provide feedback by altering the rate of uptake of  $\text{CO}_2$  by the oceans. For example, an increase in wind speeds would lead to negative feedback, tending to slow the rate of rise of atmospheric  $\text{CO}_2$ . The effect would probably be modest however, because although transfer velocities are important for the calculation of the  $\text{CO}_2$  sink, it is believed that transfer to the deep ocean is the rate-limiting step in the oceanic uptake of  $\text{CO}_2$ .

Almost all the available field data, derived at sea from the radon-deficit method and the new dual-tracer technique, and on lakes by  $\text{SF}_6$  budgeting<sup>11,13</sup>, support values for gas transfer that are within a factor of two of the Liss-Merlivat curve. The global mean transfer rate for  $\text{CO}_2$  estimated by  $^{14}\text{C}$  budgeting<sup>14</sup> also lies in this range, although substantially on the high side of the line, whereas our data at intermediate wind speeds lie on the low side (Fig. 1). The use of the Liss-Merlivat curve, in combination with data for the  $\text{CO}_2$  partial pressures in global surface water, has recently been shown to lead to substantially smaller estimates for the global ocean sink of anthropogenic  $\text{CO}_2$  than had been previously assumed<sup>1,7</sup>. Insofar as our study

TABLE 2 Gas transfer velocities

| Date<br>(1989) | $T$<br>(°C) | Hours<br>elapsed | Wind<br>speed*<br>(m s <sup>-1</sup> ) | $K_{\text{He}} - K_{\text{SF}_6}$<br>(cm h <sup>-1</sup> ) | $K_{\text{CO}_2}$ at $20^\circ\text{C}^\dagger$<br>(cm h <sup>-1</sup> ) |
|----------------|-------------|------------------|----------------------------------------|------------------------------------------------------------|--------------------------------------------------------------------------|
| 20-22 March    | 7.5         | 36.4             | 12.1 (3.9)                             | 25.0                                                       | 21.7 ± 3                                                                 |
| 22-25 March    | 7.5         | 58.2             | 17.5 (3.2)                             | 62.4                                                       | 54.2 ± 9                                                                 |
| 13, 14 October | 15.5        | 25.1             | 11.7 (2.1)                             | 22.4                                                       | 17.0 ± 6                                                                 |
| 14, 15 October | 15.5        | 29.1             | 11.9 (3.0)                             | 23.5                                                       | 17.8 ± 4                                                                 |

Determined from dual-tracer release in the North Sea.

\* Values are averages at a height of 10 m for each time period, corrected for the ship's movement. A small (~5%) adjustment has been applied to the measured wind speeds to convert from the measurement height of 18 m to the standard height of 10 m. The value in brackets is the standard deviation of a one-hour average of winds during the period.

† Errors are 1σ. Three determinations were made of each ratio except those on 14 and 15 October, when two determinations were made.



adds to confidence in the Liss-Merlivat curve, it supports this conclusion. The possibility exists, however, that gas transfer rates derived from inert-gas measurements underestimate  $\text{CO}_2$  transfer rates, for some reason not yet understood, and future studies need to address this question. □

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## Post-glacial sea-level rise from a coral record at Huon Peninsula, Papua New Guinea

John Chappell\* & Henry Polach†

\* Departments Biogeography and Geomorphology and  
† Radiocarbon Dating Laboratory, Research School of Pacific Studies,  
Australia National University, GPO Box 4, ACT 2601, Australia

DATING of coral reef terraces can provide a record of changes in sea level, which should be pronounced during the transition between glacial and interglacial periods. Cores drilled from coral reefs at Barbados in the equatorial west Atlantic<sup>1</sup> have revealed the sea-level changes that occurred during the Younger Dryas event at the end of the Last Glacial Maximum (~11,000 yr BP). It has not been known, however, whether Pacific coral reefs can grow at a rate sufficient to keep up with the rise in sea level during such a transition. Here we report results obtained from a 52-m drill core from the post-glacial reef at Huon Peninsula, Papua New Guinea, spanning the interval from 7,000 to 11,000 <sup>14</sup>C yr BP, which show that coral growth kept pace while the relative sea level rose by 50 m. Although the tectonic environment is very different from that at Barbados, the two records compare well when corrections are made for local tectonic uplift, showing that sea-level rise was similar at both locations. The rate of rise was greatest between 9,000 and 10,000 <sup>14</sup>C yr BP, corresponding to the time of the Younger Dryas.

The raised coral reef terraces at Huon Peninsula are a primary source of data on the sea level during the upper Quaternary and have been correlated isotopically with deep-sea core records<sup>2</sup>. The post-glacial reef (reef complex I) forms composite fringing reef-barrier systems, rising 5 to 23 m above sea level (a.s.l.) depending on the tectonic uplift rate, which increases from northwest to southeast along the coast<sup>3</sup>. At Sialum the Holocene lagoon is about 100–150 m wide and about 3 km long; the landward side of the fringing reef rises to 12 m and the barrier crest is up to 8 m a.s.l. The average uplift rate in the study area (Fig. 1)—based on the elevation of Pleistocene reef complex

VII which has been dated by uranium series between 118 and 140 kyr (ref. 3) and is correlated with oxygen isotope stage 5e (ref. 2)—is 1.9 m kyr<sup>-1</sup>.

A 52-m core from the emerged barrier (location, Fig. 1) was collected during an international expedition to Huon Peninsula in July 1988. The core extends through a continuous framework of coral reef. Dominant taxa, distributed fairly evenly through the core, include *Porites*, *Acropora*, *Montipora*, *Pocillopora* and *Favia Goniopora* (identifications: J.E.N. Veron). The matrix includes high-magnesium fine-textured calcite and aragonite cements, fine-grained bioclastic sediment, coral fragments, algal rinds, foraminifera and small molluscs. It contains a diverse foraminiferal assemblage of 43 taxa with no distinct compositional trend with depth in the core (C. Campbell, personal communication). Corals throughout the core are 100% aragonite, in some cases with some secondary acicular aragonite internal cement. They contrast with corals in the raised Pleistocene reef terraces which show extensive diagenesis to various forms of low-magnesium calcite. The core comes from reef that does not seem to have been subaerially exposed for any significant time, and was interpreted in the field to have grown upwards with rising post-glacial sea level. The base of the post-glacial reef was not reached owing to technical problems with drilling below 52 m.

We determined the radiocarbon ages of 27 clean coral samples, spaced fairly uniformly through the core. All samples were 100% aragonite and were free of interstitial aragonite and sediment. Dating was done at the ANU Radiocarbon Dating Laboratory using the benzene liquid-scintillation method. The laboratory is calibrated with other leading radiocarbon centres through international cross-check programmes<sup>4</sup>. Age results reported here are based on the 0.95 oxalic acid standard (National Bureau of Standards) corrected for isotope fractionation (that is, conventional D<sup>14</sup>C). A seawater correction of 400 years is subtracted; this is the same as the correction applied to Barbados coral-core samples<sup>1</sup>, and is consistent with modern (pre-1955) samples from Huon Peninsula<sup>5</sup> and slightly less than the 450-year correction adopted for eastern Australia<sup>6</sup>. Our results are listed in Table 1.

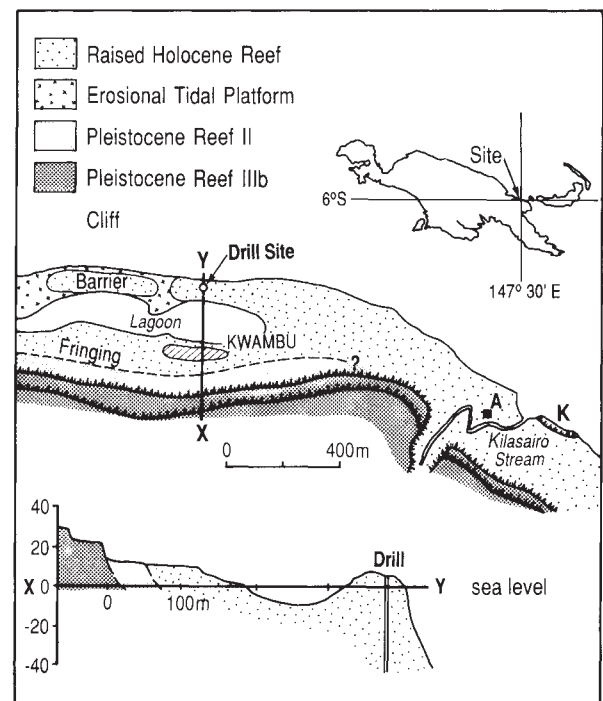


FIG. 1 Location of drill site 2 km southeast of Sialum government station on the barrier of Holocene reef complex I at Huon Peninsula.

Figure 2 shows the summary core log and an age-depth plot of the radiocarbon results. This includes some previous dates from a nearby cliff in the same reef<sup>5</sup> and from surface exposures (points A and K in Fig. 1). Figure 2 also shows age-depth data from Barbados offshore reef drill cores (corrected for Barbados uplift), plotted directly from Fairbanks<sup>1</sup>. We have also replotted the Huon core data with an uplift correction based on the average uplift rate since formation of last interglacial reef VII (oxygen isotope stage 5e). The VIIb barrier crest is 226 m a.s.l. inland of the drill site, with a uranium-series age of 118 kyr (refs 7,8). Sea level then was similar to today<sup>2</sup>. Hence, Huon uplift rate  $U$  is  $1.9 \text{ m kyr}^{-1}$ . The vertical position of a corrected point is  $H - Ut_c$  where  $H$  is actual sample elevation and  $t_c$  is the  $^{14}\text{C}$  age. Fairbanks<sup>1</sup> corrected the Barbados data in a similar way, with  $U$  (Barbados) =  $0.34 \text{ m kyr}^{-1}$ .

Uplift-corrected points plot higher in Fig. 2 than they should be because the  $^{14}\text{C}$  ages are less than 'real' ages from dendrochronology<sup>9</sup> and high-resolution uranium-series dating<sup>10</sup>. Points should be lower by  $h = U\Delta t$  where  $\Delta t$  is difference between  $^{14}\text{C}$  and 'real' age (in kyr). Relative to the Barbados data in Fig. 2, Huon points should be lower by  $\Delta h(\text{m}) = (U_H - U_B)\Delta t = 1.56\Delta t$ . If the differences between  $^{14}\text{C}$  and precise uranium-series ages found at Barbados<sup>10</sup> apply at Huon,  $\Delta h$  would increase rather smoothly from about  $-1.6\text{m}$  at 6,000 to about  $-3\text{m}$  at 11,000  $^{14}\text{C}$  yr BP. Precise uranium-series dating of the Huon core has not been done, and we have not made these corrections; the effect on the comparison is relatively minor.

Uplift-corrected Huon Peninsula and Barbados results in Fig. 2 agree remarkably well. The only divergence exceeding standard dating errors is for sea level around  $-57\text{m}$  at 10,000–10,500  $^{14}\text{C}$  years. The records track together after uplift correc-

tions, which are quite different between Barbados and Huon Peninsula ( $0.34 \text{ m kyr}^{-1}$  and  $1.9 \text{ m kyr}^{-1}$ ). This implies that both reefs are tracking the same independent factor, the post-glacial sea-level rise. The palaeo-depth of reef growth in the Huon case cannot be specified as closely as for the shallow-water *Acropora palmata* facies in Barbados corals. Between 24 and 40 m depth in the Huon core, the corals tend to have branching form, but there is nothing that suggests any significant variation of water depth throughout. These results support the sea-level curve interpreted from Barbados by Fairbanks, and support a high rate of rise between 10,000 and 9,000  $^{14}\text{C}$  yr BP. Subject to some scatter around 10,000–10,500 yr BP, results do not contradict the slower sea-level rate before 10,000  $^{14}\text{C}$  yr BP, which Fairbanks<sup>1</sup> correlates with the Younger Dryas event.

Coral reefs offshore at Barbados represent successive phases of initiation, growth and termination during post-glacial sea-level rise of 120–130 m (ref. 1). No single Barbados reef seems to have kept pace throughout the entire rise. Elsewhere, such as at the Great Barrier Reef, rapid growth began at or after 8,500  $^{14}\text{C}$  yr BP and often lagged behind rising sea level<sup>11</sup>. Our results show that reef growth at Huon Peninsula kept pace with sea level from at least 11,100  $^{14}\text{C}$  yr BP; further drilling is required to discover whether this occurred through the entire post-glacial rise. The rate of relative sea-level rise at Huon Peninsula is offset by tectonic uplift of  $1.9 \text{ m kyr}^{-1}$ , which is small compared with the average reef growth of  $12.7 \text{ m}$  per 1,000  $^{14}\text{C}$  years. If the relationship between  $^{14}\text{C}$  and uranium timescales discovered at Barbados<sup>10</sup> is confirmed by similar work at Huon Peninsula, the time interval represented by our core would be  $\sim 5,000$  years, giving a mean reef growth rate of  $10 \text{ m kyr}^{-1}$ . The maximum rate occurred between 9,000 and 10,000  $^{14}\text{C}$  yr BP, when the

TABLE 1 Radiocarbon dates

| ANU lab number | Depth below top (m) | Coral genus* | $^{14}\text{C}$ age (yr BP)† |
|----------------|---------------------|--------------|------------------------------|
| 6758           | 1.2                 | Por.         | $7,400 \pm 80$               |
| 6770           | 3.4                 | Por.         | $7,800 \pm 80$               |
| 6759           | 4.4                 | Pocil.       | $7,980 \pm 70$               |
| 6771           | 6.2                 | Por.         | $8,160 \pm 70$               |
| 6760           | 7.7                 | Por.         | $8,100 \pm 80$               |
| 6772           | 9.0                 | Acrop.       | $8,330 \pm 80$               |
| 6774           | 10.6                | Por.         | $8,130 \pm 80$               |
| 6761           | 14.2                | Por.         | $8,600 \pm 70$               |
| 6775           | 15.4                | Por.         | $8,930 \pm 70$               |
| 6762           | 17.4                | Acrop.       | $9,000 \pm 70$               |
| 6776           | 20.7                | Por.         | $9,010 \pm 70$               |
| 6763           | 21.3                | Gonio.       | $9,290 \pm 70$               |
| 6777           | 22.8                | Monti.       | $9,370 \pm 70$               |
| 6778           | 23.7                | Monti.       | $9,310 \pm 100$              |
| 6764           | 25.3                | Monti.       | $9,690 \pm 70$               |
| 6779           | 26.1                | Por.         | $9,460 \pm 60$               |
| 6781           | 29.2                | Por.         | $9,770 \pm 80$               |
| 6765           | 30.6                | Monti.       | $9,870 \pm 70$               |
| 6782           | 32.0                | Por.         | $9,900 \pm 90$               |
| 6783           | 33.1                | Por.         | $9,880 \pm 70$               |
| 6784           | 34.0                | Por.         | $9,640 \pm 80$               |
| 6766           | 36.8                | Por.         | $10,100 \pm 70$              |
| 6785           | 38.4                | Por.         | $10,260 \pm 80$              |
| 6773           | 39.8                | Por.         | $10,350 \pm 90$              |
| 6786           | 42.5                | Acrop.       | $11,130 \pm 80$              |
| 6767           | 42.7                | Monti.       | $10,820 \pm 70$              |
| 6787           | 45.2                | Por.         | $10,840 \pm 80$              |
| 6768           | 50.0                | Pocil.       | $11,110 \pm 90$              |
| 6769           | 51.4                | Monti.       | $11,480 \pm 90$              |

\* Identifications by J. E. N. Veron: Por., *Porites*; Pocil., *Pocillopora*; Monti., *Montipora*; Acrop., *Acropora*; Gonio., *Goniopora*.

† 5,568-yr half-life, corrected for  $^{13}\text{C}$  content. Results plotted in Fig. 2 have 400-yr seawater correction subtracted.

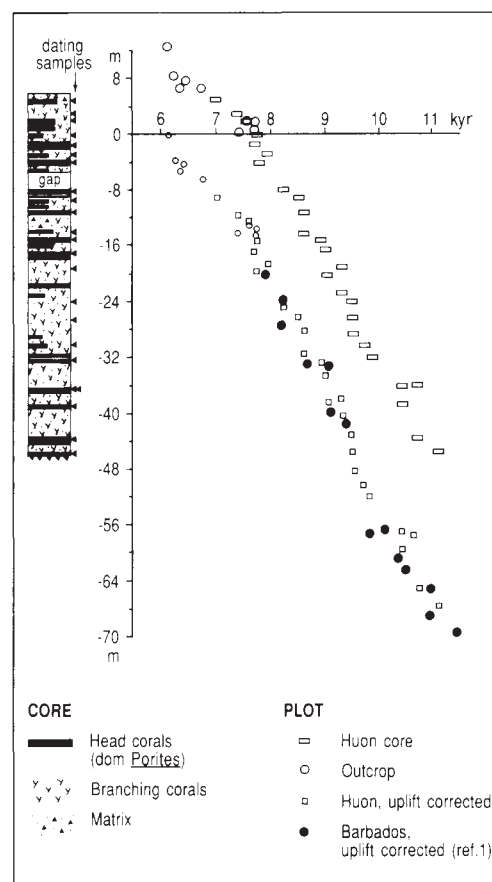


FIG. 2 Summary core log and age-depth plots of radiocarbon results. Upper age-depth series is for samples uncorrected for uplift; lower series is corrected for uplift and is compared with Barbados results<sup>1</sup>.

Huon reef grew  $\sim 20$  m; this is  $\sim 13$  m kyr $^{-1}$  after conversion from  $^{14}\text{C}$  to uranium-series timescales, about twice the  $^{14}\text{C}$ -based rate of the Great Barrier Reef<sup>11</sup>. The concordance of Huon and Barbados results, after uplift correction, indicates that Pacific as well as Atlantic reefs can provide good data on sea level at sites favourable for rapid and sustained growth. □

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## Possible solar noble-gas component in Hawaiian basalts

Masahiko Honda\*, Ian McDougall\*,  
Desmond B. Patterson\*, Anthony Doulgeris\*  
& David A. Clague†

\* Research School of Earth Sciences, The Australian National University,  
GPO Box 4, Canberra, ACT 2601, Australia

† US Geological Survey, Menlo Park, California 94025, USA

THE noble-gas elemental and isotopic composition in the Earth is significantly different from that of the present atmosphere, and provides an important clue to the origin and history of the Earth and its atmosphere. Possible candidates for the noble-gas composition of the primordial Earth include a solar-like component, a planetary-like component (as observed in primitive meteorites) and a component similar in composition to the present atmosphere. In an attempt to identify the contributions of such components, we have measured isotope ratios of helium and neon in fresh basaltic glasses dredged from Loihi seamount and the East Rift Zone of Kilauea<sup>1–3</sup>. We find a systematic enrichment in  $^{20}\text{Ne}$  and  $^{21}\text{Ne}$  relative to  $^{22}\text{Ne}$ , compared with atmospheric neon. The helium and neon isotope signatures observed in our samples can be explained by mixing of solar, present atmospheric, radiogenic and nucleogenic components. These data suggest that the noble-gas isotopic composition of the mantle source of the Hawaiian plume is different from that of the present atmosphere, and that it includes a significant solar-like component. We infer that this component was acquired during the formation of the Earth.

Non-atmospheric Ne isotope ratios and coexisting primordial  $^3\text{He}$  have previously been identified in basaltic glasses and volcanic gases from mid-ocean ridges<sup>4–6</sup>, from the Hawaiian<sup>5,7</sup> and Yellowstone<sup>8</sup> hotspots, and from diamonds<sup>9,10</sup>. In particular, the Ne results<sup>5</sup> from mid-ocean-ridge basalts (MORBs) lie on a straight line in a three-isotope plot of  $^{20}\text{Ne}/^{22}\text{Ne}$  and  $^{21}\text{Ne}/^{22}\text{Ne}$ , with one endmember being atmospheric Ne and the other endmember enriched in  $^{20}\text{Ne}$  and  $^{21}\text{Ne}$  relative to  $^{22}\text{Ne}$  (Fig. 1a). To determine whether a similar Ne correlation can be identified in samples from a so-called undepleted mantle source, we have measured Ne isotope ratios in 18 samples from the Loihi seamount and the East Rift Zone of Kilauea; the results are shown in Figs 1a, b. The Ne results from the Loihi-Kilauea (L-K) samples form a linear array, which has a distinctly different slope from both the MORB line and the mass-fractionation line, all lines passing through atmospheric Ne.

Individual samples are enriched in  $^{20}\text{Ne}$  and  $^{21}\text{Ne}$  by as much as 16% with respect to atmospheric ratios. Similar Ne isotope anomalies have been reported by Sarda *et al.*<sup>5</sup> in two samples from the Loihi seamount, whereas the rest of their seven samples showed atmospheric Ne isotope ratios, within the uncertainties.

The L-K samples with atmosphere-like Ne generally have large Ne gas concentrations, consistent with a significant degree of atmospheric contamination of the magmas before eruption<sup>11</sup>. The correlation lines shown in Fig. 1 can be interpreted as mixing lines between present atmospheric Ne as a common endmember, and non-atmospheric components in the L-K and MORB samples, which are derived from mantle sources characterized by different proportions of non-atmospheric components.

Non-atmospheric neon components that have been identified include solar, planetary, nucleogenic and spallogenic<sup>12</sup>. None of these, however, lie directly on either the L-K or MORB correlation lines. Thus, to explain the enrichment of  $^{21}\text{Ne}$  and  $^{20}\text{Ne}$  in the mantle sources for the L-K and MORB samples, it is necessary to mix at least two distinct non-atmospheric components. The two most likely candidates are nucleogenic and

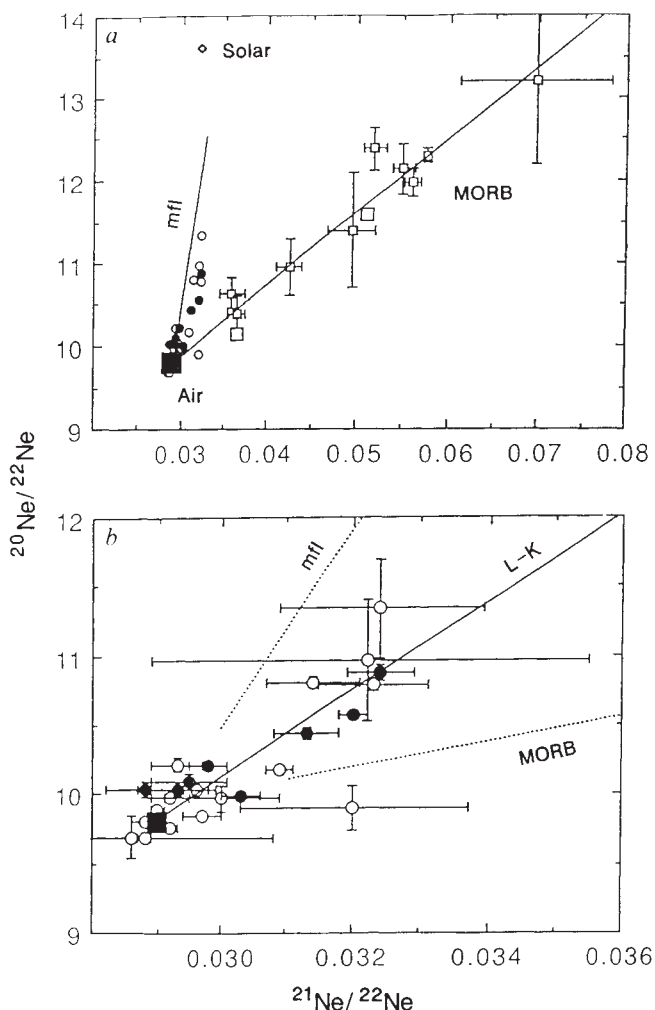


FIG. 1 Three-isotope plots for neon. Atmospheric (Air) and solar values are shown, as well as the mass-fractionation line (mfl) for atmospheric Ne. Small open squares show results from MORBs<sup>5</sup>. Large open squares are our unpublished MORB data (uncertainties within symbol). The MORB correlation line is as given by Sarda *et al.*<sup>5</sup>. Open and filled circles are data from 18 Loihi and Kilauea basalt glass samples, respectively, shown a, without uncertainties and b, with uncertainties ( $1\sigma$ ). The solid line in b is the best-fit regression line calculated from results from 26 measurements on 18 samples, and includes 15 results based on step-heating experiments and 11 datum points derived from fusing samples directly at 1,500 °C.



TABLE 1 He and Ne isotopic compositions of quenched basaltic glasses from Loihi seamount and East Rift Zone of Kilauea

| Sample*                            | $^4\text{He}$<br>( $10^{-9} \text{ cm}^3 \text{ STP g}^{-1}$ ) | $^3\text{He}/^4\text{He}$<br>( $10^{-6}$ ) | $^{22}\text{Ne}$<br>( $10^{-12} \text{ cm}^3 \text{ STP g}^{-1}$ ) | $^{20}\text{Ne}/^{22}\text{Ne}$ | $^{21}\text{Ne}/^{22}\text{Ne}$ | $^{21}\text{Ne}_n^\dagger$<br>( $10^{-15} \text{ cm}^3 \text{ STP g}^{-1}$ ) | $^{21}\text{Ne}_n/^{4}\text{He}^\dagger$<br>( $10^{-7}$ ) | $^{22}\text{Ne}_s/^{21}\text{Ne}_n^\dagger$ |
|------------------------------------|----------------------------------------------------------------|--------------------------------------------|--------------------------------------------------------------------|---------------------------------|---------------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------|---------------------------------------------|
| Loihi KK-29-4-a<br>(1.088 g; 3.4%) |                                                                |                                            |                                                                    |                                 |                                 |                                                                              |                                                           |                                             |
| 600 °C                             | 47 (1) [0.27]                                                  | 44.0 (8)                                   | 1.0 (1) [0.34]                                                     | 10.97 (44)                      | 0.0322 (33)                     |                                                                              |                                                           |                                             |
| 1,500 °C                           | 156 (1) [0.27]                                                 | 45.7 (8)                                   | 19.0 (1) [0.34]                                                    | 10.81 (4)                       | 0.0314 (7)                      |                                                                              |                                                           |                                             |
| Total                              | 203 (1)                                                        | 45.3 (6)                                   | 20.0 (2)                                                           | 10.82 (4)                       | 0.0314 (6)                      | 32 (11)                                                                      | 1.6 (6)                                                   | 167 (60)                                    |
| Loihi KK-29-4-b<br>(2.020 g; 3.4%) |                                                                |                                            |                                                                    |                                 |                                 |                                                                              |                                                           |                                             |
| 600 °C                             | 145 (1) [0.28]                                                 | 40.9 (6)                                   | 30.0 (3) [0.50]                                                    | 9.80 (2)                        | 0.0288 (2)                      |                                                                              |                                                           |                                             |
| 700 °C                             | 98 (1) [0.28]                                                  | 38.2 (5)                                   | 3.9 (2) [0.50]                                                     | 9.97 (1)                        | 0.0300 (9)                      |                                                                              |                                                           |                                             |
| 1,500 °C                           | 355 (4) [0.28]                                                 | 37.4 (5)                                   | 19.6 (2) [0.50]                                                    | 10.79 (4)                       | 0.0323 (8)                      |                                                                              |                                                           |                                             |
| Total                              | 598 (4)                                                        | 38.4 (3)                                   | 53.6 (4)                                                           | 10.17 (2)                       | 0.0302 (3)                      | 51 (13)                                                                      | 0.9 (2)                                                   | 104 (27)                                    |
| Loihi KK-20-2<br>(1.630 g; 5.2%)   |                                                                |                                            |                                                                    |                                 |                                 |                                                                              |                                                           |                                             |
| 600 °C                             | 200 (2) [0.42]                                                 | 39.4 (5)                                   | 43 (1) [4.0]                                                       | 9.97 (2)                        | 0.0292 (3)                      |                                                                              |                                                           |                                             |
| 1,500 °C                           | 767 (8) [0.42]                                                 | 36.9 (5)                                   | 90 (1) [4.0]                                                       | 10.04 (2)                       | 0.0296 (2)                      |                                                                              |                                                           |                                             |
| Total                              | 968 (8)                                                        | 37.4 (4)                                   | 133 (1)                                                            | 10.01 (1)                       | 0.0295 (1)                      | 41 (18)                                                                      | 0.4 (2)                                                   | 180 (77)                                    |
| Loihi KK-26-4<br>(1.074 g; 3.5%)   | 1,098 (73) [0.28]                                              | 30.5 (4)                                   | 146 (3) [3.2]                                                      | 10.18 (2)                       | 0.0309 (2)                      | 225 (30)                                                                     | 2.0 (3)                                                   | 65 (9)                                      |
| Kilauea 1701<br>(1.006 g; 0.2%)    | 9.4 (5) [0.48]                                                 | 19.1 (1.1)                                 | 108 (6) [6.1]                                                      | 9.99 (3)                        | 0.0303 (4)                      | 125 (42)                                                                     | 130 (40)                                                  | 43 (16)                                     |
| Kilauea 1712-a<br>(1.062 g; 0.5%)  | 548 (5) [0.36]                                                 | 19.8 (3)                                   | 40.0 (1.3) [5.6]                                                   | 10.88 (6)                       | 0.0324 (5)                      | 100 (18)                                                                     | 1.8 (3)                                                   | 113 (22)                                    |
| Kilauea 1712-b<br>(1.990 g; 0.5%)  |                                                                |                                            |                                                                    |                                 |                                 |                                                                              |                                                           |                                             |
| 600 °C                             | 102 (1) [0.24]                                                 | 21.5 (3)                                   | 13.6 (3) [1.6]                                                     | 10.44 (4)                       | 0.0313 (5)                      |                                                                              |                                                           |                                             |
| 1,500 °C                           | 284 (3) [0.24]                                                 | 19.1 (3)                                   | 36.1 (3) [1.6]                                                     | 10.57 (2)                       | 0.0320 (2)                      |                                                                              |                                                           |                                             |
| Total                              | 386 (3)                                                        | 19.7 (2)                                   | 49.7 (4)                                                           | 10.53 (2)                       | 0.0318 (2)                      | 110 (9)                                                                      | 2.8 (2)                                                   | 87 (8)                                      |
| Kilauea 1706<br>(1.062 g; 0%)      | 10.4 (1) [0.36]                                                | 18.3 (8)                                   | 94.4 (1.9) [5.7]                                                   | 10.05 (3)                       | 0.0305 (4)                      | 125 (33)                                                                     | 120 (30)                                                  | 50 (13)                                     |
| Atmosphere‡                        |                                                                | 1.4                                        |                                                                    | 9.80                            | 0.0290                          |                                                                              |                                                           |                                             |
| Solar‡                             |                                                                | 400                                        |                                                                    | 13.6                            | 0.032                           |                                                                              |                                                           |                                             |
| Nucleogenic§                       |                                                                |                                            |                                                                    | 2.5                             | 32                              |                                                                              |                                                           |                                             |

He and Ne, extracted from 1 to 2 g samples of thin (2–5 mm) glassy rims of dredged pillow basalts, were analysed using a VG5400 mass spectrometer fitted with a Nier-type electron bombardment source. Rare phenocrysts were removed from the glass. Helium was separated from Ne on activated charcoal at 50 K and measured relative to the Yellowstone 'Mud Volcano' standard ( $^3\text{He}/^4\text{He}=2.28\times 10^{-5}$ ). Neon was separated from the heavier noble gases at 125 K and measured relative to atmospheric neon. Results were corrected for small interferences from  $^{40}\text{Ar}^{2+}$ ,  $\text{CO}_2^{2+}$  and  $\text{H}_2^{18}\text{O}^+$ . The hot blanks (1,500 °C) for  $^4\text{He}$  and  $^{22}\text{Ne}$  were in the range  $3\text{--}6\times 10^{-10}$  and  $0.4\text{--}6\times 10^{-12} \text{ cm}^3 \text{ STP}$ , respectively. An average of the hot blank measured before and after each sample was used for the hot-blank subtraction. Blank  $^4\text{He}$  and  $^{22}\text{Ne}$  amounts for individual runs are shown in square brackets. Blank He isotope ratio was assumed to be atmospheric. The average Ne isotope ratios of the blanks, determined from 40 runs, were atmospheric within uncertainties ( $^{20}\text{Ne}/^{22}\text{Ne}=9.92\pm 0.09$ ,  $^{21}\text{Ne}/^{22}\text{Ne}=0.0291\pm 0.0005$ ), and these values were used for Ne hot-blank subtraction. Quoted  $1\sigma$  errors on the last significant figure(s), shown in parentheses, include uncertainties in the correction factors for mass discrimination and sensitivity determined by repeated analysis of the standard gases, as well as uncertainties in the blank correction and interference corrections in the case of Ne. Unless extraction temperatures for step-heating are tabulated, samples were totally fused at 1,500 °C. Technical details will be published elsewhere. To demonstrate the reproducibility of our Ne isotope measurements, samples Loihi KK-29-4 and Kilauea 1712, which show the highest anomalies in our data set, were measured in duplicate.

\* Except for one transitional basalt glass sample, KK-26-4, all samples are tholeiitic in composition. Sample weight used for noble gas analysis and vesicularity in % ( $\text{cm}^3$  per total  $\text{cm}^3$ ) are shown in brackets.

† The stated compositions of the three Ne endmembers; atmospheric, nucleogenic and solar, were used to calculate nucleogenic  $^{21}\text{Ne}$  ( $^{21}\text{Ne}_n$ ) and solar  $^{22}\text{Ne}$  ( $^{22}\text{Ne}_s$ ) from the observed Ne isotope ratios. The three endmember mixing equations used for the deconvolution are  $(^{21}\text{Ne}/^{22}\text{Ne})_{\text{observed}} = k(^{21}\text{Ne}/^{22}\text{Ne})_{\text{atmospheric}} + l(^{21}\text{Ne}/^{22}\text{Ne})_{\text{nucleogenic}} + m(^{21}\text{Ne}/^{22}\text{Ne})_{\text{solar}}$ , where  $k$ ,  $l$  and  $m$  are the fractional contributions of each endmember to the reference isotope  $^{22}\text{Ne}$ ,  $k+l+m=1$ , and  $^4\text{He} = ^{20}\text{Ne}$  or  $^{21}\text{Ne}$ .

‡ ref. 12 and references therein.

§ ref. 13.

solar Ne. Nucleogenic  $^{21}\text{Ne}$ , produced by the  $^{18}\text{O}(\alpha, n)^{21}\text{Ne}$  and  $^{24}\text{Mg}(n, \alpha)^{21}\text{Ne}$  reactions from local decay of U and Th, elevates  $^{21}\text{Ne}/^{22}\text{Ne}$  ratios, whereas there are no known nucleogenic reactions that produce significant amounts of  $^{20}\text{Ne}$  (ref. 12). Solar Ne is the only known component that has a  $^{20}\text{Ne}/^{22}\text{Ne}$  ratio (13.6) greater than the atmospheric value (9.8) and greater than the  $^{20}\text{Ne}/^{22}\text{Ne}$  ratios observed in the L-K and MORB samples (Fig. 1a). We therefore suggest, as a working hypothesis, that the Ne compositions of L-K and MORB samples arise from the mixing of three Ne components: solar, nucleogenic and atmospheric (Table 1).

By defining a unique Ne isotopic composition for each of these three components, we can deconvolve the relative contribution of each of the components in the samples. For this purpose, we consider only the L-K samples with Ne isotope ratios differing by more than two standard deviations from atmospheric values, as listed in Table 1. We estimate nucleogenic  $^{21}\text{Ne}$  (hereafter  $^{21}\text{Ne}_n$ ) and solar  $^{22}\text{Ne}$  (hereafter  $^{22}\text{Ne}_s$ ) from the L-K samples (see footnote to Table 1) and compare the amounts of  $^{21}\text{Ne}_n$  and  $^4\text{He}$ . We make the reasonable assumption that all of the observed  $^4\text{He}$  is produced from the decay of U and Th. Calculated  $^{21}\text{Ne}_n/^4\text{He}$  ratios from the majority of L-K samples (Table 1) are close to the estimated production ratio of  $1\times 10^{-7}$  (ref. 13). This is consistent with the hypothesis that

the excess  $^{21}\text{Ne}$  and most of the  $^4\text{He}$  have been produced in the Earth by radioactive processes over geological time. The similarity between the estimated production ratio and the  $^{21}\text{Ne}_n/^4\text{He}$  ratios calculated for the L-K samples further indicates that there has been no substantial elemental fractionation between He and Ne since the mantle source region for the L-K samples became isolated. As isotope fractionation is likely to occur only under more extreme physical conditions than elemental fractionation, this lack of evidence for elemental fractionation suggests that isotope fractionation of Ne is unlikely to have occurred. The  $^{21}\text{Ne}_n/^4\text{He}$  results from samples Kilauea 1701 and 1706 differ significantly from the production ratio; these can be explained by preferential He loss from the samples before and/or during eruption, as indicated by their low He abundances compared with He abundances in other samples.

If the Earth's primordial composition was solar, then we expect to see a correlation between the Ne and He isotope systematics. This is because the bulk of the Ne, after the subtraction of atmospheric Ne, would be primordial, and  $^3\text{He}$  is believed to be primordial<sup>14</sup>, and both  $^{21}\text{Ne}_n$  and  $^4\text{He}$  are the products of radioactive processes having a unique  $^4\text{He}/^{21}\text{Ne}_n$  production ratio<sup>13</sup>.

Under this hypothesis, the slopes of the Ne mixing lines in Fig. 1 are a function of the ratio of solar Ne to nucleogenic

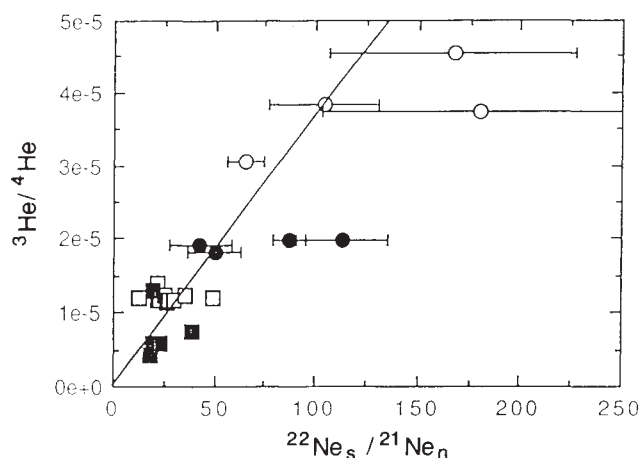


FIG. 2 Measured  $^3\text{He}/^4\text{He}$  ratios plotted against the calculated solar  $^{22}\text{Ne}_s/\text{nucleogenic } ^{21}\text{Ne}_n$  results for the L-K (circles), MORB (open squares)<sup>5</sup>, and diamond samples (filled squares)<sup>10</sup>. From the slope of the best-fit line we estimate  $^{3}\text{He}/^{22}\text{Ne}_s$  ratio of  $3.7 \pm 1.4$ , assuming a  $^{21}\text{Ne}_n/^4\text{He}$  production ratio of  $1 \times 10^{-7}$ .

$^{21}\text{Ne}$  in the mantle, and the amount of  $^{21}\text{Ne}_n$  is a function of the U and Th content of the sources. Thus, the steeper slope of the L-K line compared with the MORB line in Fig. 1 indicates that the L-K source is enriched in solar Ne with respect to  $^{21}\text{Ne}_n$  in comparison with the MORB source, and must therefore have a higher solar Ne to U+Th ratio. In a similar fashion, the  $^3\text{He}/^4\text{He}$  ratios observed in L-K samples are higher than MORB samples ( $(R/R_a)_{\text{L-K}} = 13\text{--}35$ ,  $(R/R_a)_{\text{MORB}} \approx 9$ ; where  $R/R_a = (^3\text{He}/^4\text{He})/(^3\text{He}/^4\text{He})_{\text{air}}$ ; this work and refs 5, 7, 14–16), indicating that the ratio of primordial He to U+Th for the L-K source is, like the solar Ne to U+Th ratio, higher than that of the MORB source. In this context, we point out that Ne isotope results obtained from fluids associated with the Yellowstone hotspot<sup>8</sup> lie on a mixing line quite similar to that of the L-K samples, and that the  $^3\text{He}/^4\text{He}$  ratios observed in Yellowstone samples are as much as 16 times the air value.

Calculated ratios of primordial  $^{22}\text{Ne}_s$  to  $^{21}\text{Ne}_n$  from the L-K, MORB<sup>5</sup> and diamond samples<sup>10</sup> are plotted against measured  $^3\text{He}/^4\text{He}$  ratios in Fig. 2. Although there is some scatter of the data, especially for the L-K samples, because of the large corrections for atmospheric contamination, there is a clear correlation between  $^3\text{He}/^4\text{He}$  and  $^{22}\text{Ne}_s/^{21}\text{Ne}_n$  ratios. Because both axes represent isotope ratios, the slope of a line in such a diagram is independent of any elemental fractionation. Given that both  $^4\text{He}$  and  $^{21}\text{Ne}_n$  are products of the radioactive processes associated with the decay of U and Th, the slope  $S$  of the line is a function of the  $^{21}\text{Ne}_n/^4\text{He}$  production ratio and the primordial  $^3\text{He}/^{22}\text{Ne}_s$  ratio, being given by  $S = (^3\text{He}/^{22}\text{Ne}_s)(^{21}\text{Ne}_n/^4\text{He})$ . Taking the estimated  $^{21}\text{Ne}_n/^4\text{He}$  production ratio of  $1 \times 10^{-7}$ , we calculate a primordial  $^3\text{He}/^{22}\text{Ne}_s$  ratio of  $3.7 \pm 1.4$ , which is indistinguishable from the solar ratio of 3.3 observed in solar wind<sup>12</sup>, but is distinctly different from the planetary ratio of 0.3 observed in meteorites<sup>12</sup>.

Hence, our working hypothesis that three-endmember mixing can account for the Ne isotope anomalies seems to be internally consistent. Our three-endmember mixing model is only one of a number of possible interpretations (see, for example, Sarda *et al.*<sup>5</sup>), but for reasons that will be discussed elsewhere, we believe that these alternative interpretations are inconsistent with our data. Thus, we conclude that the noble-gas composition in the mantle is distinctly different from that of the present atmosphere and that it involves a significant solar component. We further infer that the primordial noble-gas composition of the Earth is likely to be solar, and that evidence for this solar component remains in the mantle, even though there are strong reasons for believing there was extensive outgassing of the Earth soon after its formation (see, for example, ref. 17). □

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## Evidence for a regional-scale fluid loss event during mid-crustal metamorphism

Bruce Yardley, Simon H. Bottrell & R. A. Cliff

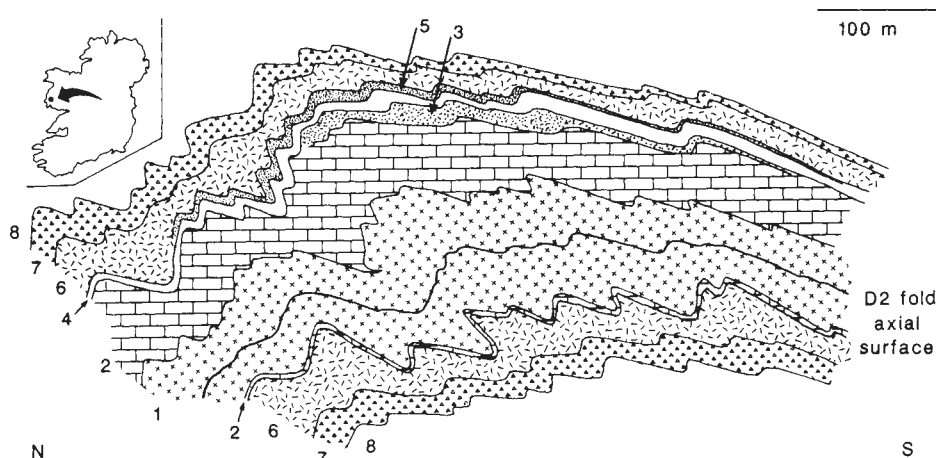
Department of Earth Sciences, University of Leeds, Leeds LS2 9JT, UK

**HOW fluid is released from the deep crust during metamorphism remains controversial. Whereas some authors advocate large-scale fluid fluxes due to convective circulation of metamorphic fluid<sup>1</sup>, others consider that only one-way flow is possible<sup>2,3</sup>. Furthermore, some flow models imply that fluid moves across, and largely independently of, sedimentary layering<sup>1,2</sup>, whereas many fluid-based studies find evidence for focusing of flow by particular lithologies<sup>2,4–7</sup>. Here we report a study of metamorphic fluid flow on a regional, rather than an outcrop scale, using a combination of field geology, petrology and theoretical and isotope geochemistry. We identify metasomatic lithologies at the top of a dolomitic marble unit, which must have drawn in water over a kilometre scale from adjacent pelitic formations; we suggest that this flow was part of a single, major fluid loss event near the metamorphic peak. Fluid followed the metasomatic layers to structural highs where it broke out into adjacent schists to form skarns. This fluid loss may have changed the rheology of the rock involved and terminated regional folding.**

The area investigated is in Connemara, western Ireland, where a late Precambrian (Dalradian) succession of sediments has undergone a complex history of folding and metamorphism<sup>8</sup>, culminating in a low-pressure, high-temperature metamorphism with accompanying folding (D3) that was related to the emplacement of calc-alkaline intrusions exposed to the south of the metasediments. The sedimentary sequence is dominated by psammites, quartzites and pelites with some nearly pure calcite marbles and interbedded orthoamphibolites<sup>9</sup>. At the base of the succession, outcropping only in the hinge of an early (D2) isoclinal fold which broadly follows the central E–W axis of Connemara, lies the Connemara Marble formation (CMF). The CMF includes dolomitic marbles, calc-silicates and ophicarbonate rocks derived from former dolomitic limestones<sup>10</sup> and all the major occurrences of the CMF experienced similar maximum temperatures and pressures of metamorphism<sup>8</sup>,  $T = 650 \pm 40^\circ\text{C}$  and  $P = 4\text{--}5.5$  kbar.

At one inlier of the CMF in Glencoaghan (Irish grid ref. L805485) we could map the different lithologies in the CMF, and these are shown in section in Fig. 1. They include both isochemically metamorphosed sediments and layers of extreme composition that are probably of metasomatic origin. At Glencoaghan we identified two metasomatic rock types; both map as 'beds'. They are the diopside rock at the top of the formation in contact with overlying pelite, and a serpentine (formerly

FIG. 1 North-south sketch section of the Glencoaghan inlier, central Connemara (see inset), illustrating the disposition of metasomatic lithologies (stippled ornaments) in the refolded fold structure. Rock types are numbered from oldest to youngest as follows: 1, calc-silicate rock; 2, calcite-tremolite marble; 3, serpentine marble; 4, quartzite; 5, metasomatic diopside rock (1-5 all in the Connemara Marble formation); 6, pelite and psammite (Barnanoraun formation); 7, psammite (Cleggan Boulder Bed formation); 8, massive quartzite.



forsterite<sup>10</sup>)-phlogopite green marble beneath the diopside rock, separated from it by a thin quartzite. The diopside rock is up to 3 m thick and recurs in the same structural position for at least 12 km to the west. It is very coarse grained (often several centimetres) and has the bulk composition of nearly pure diopside although minor retrograde tremolite and calcite are present; it is cut distinctively by thin quartz veins. Back-scattered electron and cathodoluminescence imaging reveals that the diopside crystals have homogeneous cores ( $X_{Mg}=0.98$ ) with corroded outlines, enclosed in overgrowths with fine-scale oscillatory growth zoning ( $X_{Mg}=0.88-0.98$ ). This zoning style indicates growth conditions far removed from equilibrium. Both this bed and the underlying green marble (<5 m) developed assemblages that are only stable in the presence of a water-rich fluid<sup>11</sup>, despite the fact that the reactions that produced them release CO<sub>2</sub>-rich fluid. Marbles a few metres deeper in the succession contain assemblages (such as tremolite + calcite + quartz or tremolite + calcite + dolomite) requiring a fluid richer in CO<sub>2</sub><sup>11</sup>. The infiltration of water to form the metasomatic rocks occurred only at high grade. At lower temperature, metasomatic talc or tremolite would form instead of diopside, and even if these then underwent thermal breakdown with further heating, the resulting bed could not have the bulk composition of diopside, as now seen for the diopside rock.

Our interpretation of this succession is that water penetrated the CMF from nearby schists. The top marble bed was infiltrated by quartz-saturated water to form diopside rock, but as the water penetrated deeper it lost silica so that quartz-undersaturated, forsterite-bearing assemblages developed. The core of the marble was not infiltrated to the same extent, and the fluid remained internally buffered for CO<sub>2</sub>.

To test this hypothesis we have carried out oxygen isotope analyses on calcite from marble, on quartz separates and vein quartz from silicate rocks and marbles, and on diopside from the diopside rock. In addition, strontium isotope analyses have been performed on a suite of whole-rock samples, and corrected to give the <sup>87</sup>Sr/<sup>86</sup>Sr values at the time of metamorphism, 480 Myr ago<sup>8,12</sup>.

Our results, with additional Sr data from the literature, are summarized in Fig. 2. Pure calcite marbles from the middle Dalradian Lakes Marble formation (LMF) to the east of Glencoaghan largely display a heavy O signature and have a Sr composition appropriate for their original limestone parents. The LMF probably indicates the original composition of the parental CMF limestones. In contrast, pelites from various formations have distinctive values for both parameters. Marbles from the CMF fall in an intermediate field, consistent with variable infiltration of pelite-derived fluid into primary marble

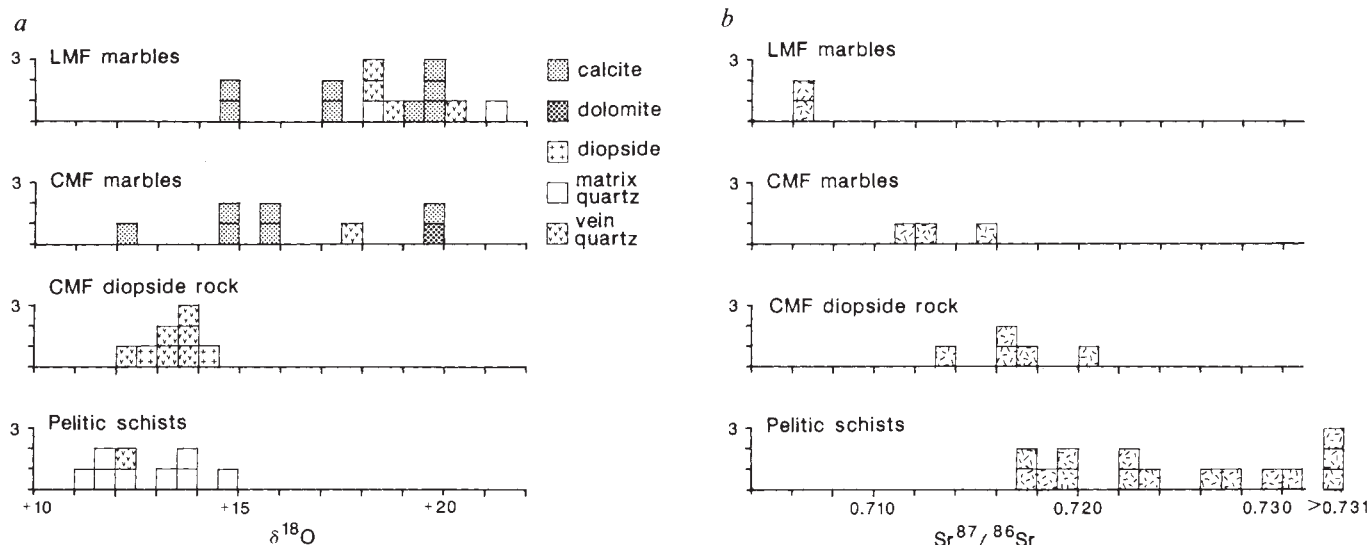


FIG. 2 Histograms illustrating the O and Sr isotope variation between calcite marbles of the LMF, less pure calcite and dolomite marbles of the CMF, diopside rock (CMF) and interbedded Dalradian pelites. a,  $\delta^{18}O$  results on

mineral separates of quartz, calcite, dolomite and diopside. b,  $^{87}Sr/^{86}Sr$  at 480 Myr; pelite data from refs 13, 21.



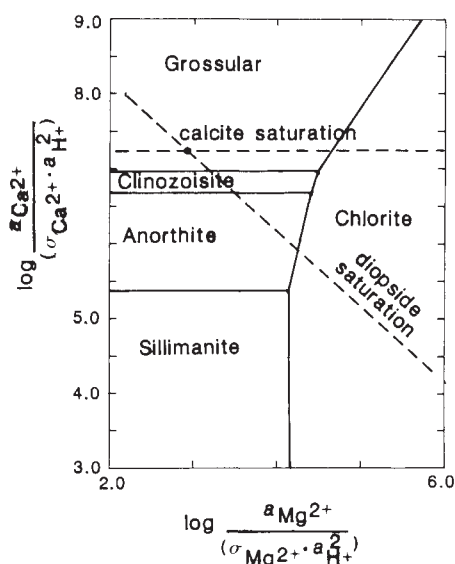


FIG. 3 Log activity diagram of the stability relationships between minerals representative of psammitic host rocks (anorthite, chlorite, sillimanite) and those representative of skarns (clinzoisite, grossular) in the presence of a water-rich fluid ( $X_{\text{CO}_2}=0.1$ ). Quartz saturation is assumed. The diagonal dashed line represents fluids also saturated with diopside, the horizontal one defines calcite saturation.  $P$ - $T$  conditions are 600 °C and 5 kbar; the diagram is modified from Bower *et al.*<sup>17</sup>. The  $\sigma$  parameter makes the small correction necessary for the effect of  $\text{CO}_2$  on the activity of water<sup>20</sup>.

similar to LMF. Diopside rock is isotopically more nearly equilibrated with the pelites, supporting their role as a source of infiltrating water; contemporaneous granites had  $^{87}\text{Sr}/^{86}\text{Sr} < 0.715$  at this time<sup>13</sup>, ruling them out as a fluid source.

It is difficult to make a reliable estimate of the ratio of water to rock during the metasomatic event that formed the diopside rock—although both the Sr and O isotopic compositions of the diopside are intermediate between those of parental marble and adjacent pelite, the uncertainties in the calculated ratios are large. From the Sr data (Fig. 2) we estimate an integrated water:rock ratio near 4:1 by assuming that the Sr content of fluid and marble were similar, but in reality the Sr content of the fluid is essentially unknown, so there is an order-of-magnitude uncertainty. Likewise, a ratio based on the O isotope data depends on assumptions about the primary composition of the layer and the fractionation of oxygen during decarbonation<sup>14</sup>. The result obtained will depend on the assumptions, and not provide a test for them. As it is the uniform monomineralic composition of the diopside rock that identifies it as metasomatic, we have estimated a water:rock ratio from the amount of  $\text{SiO}_2$  that must be added to initial quartz-bearing dolomitic marble to produce the monomineralic diopside composition

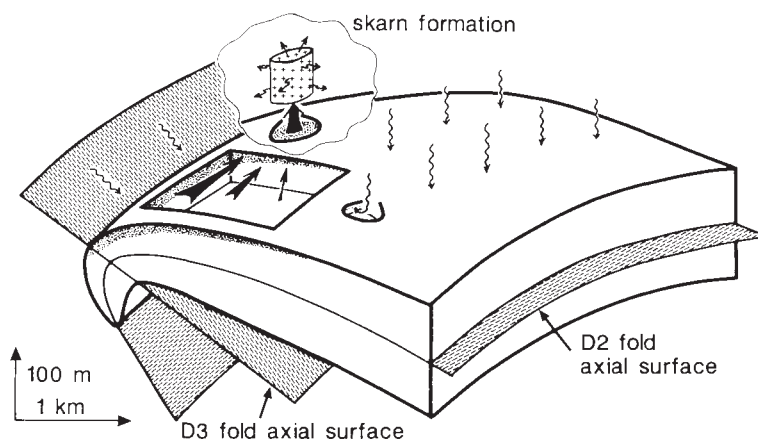
(~55 wt%  $\text{SiO}_2$ ). We assumed that homogeneous diopside cores grew from dolomite and quartz in the parental marble, but zoned rims grew from metasomatic addition of  $\text{SiO}_2$  to the excess dolomite remaining. These zoned portions comprise 10–40% of the diopside in different samples; using a conservative value of 10% for the amount of metasomatic diopside and  $6.5 \text{ g kg}^{-1}$  for the available  $\text{SiO}_2$  in the infiltrating quartz-saturated fluid<sup>15</sup> gives a ratio of 8.5:1, which should probably be viewed as a minimum.

Dehydration of pelite with 25% muscovite yields only ~1%  $\text{H}_2\text{O}$ , so to infiltrate a 1-m-thick bed of diopside rock with 8.5 times its mass of water requires all the fluid given off by dehydration of an 850-m-thick bed of pelite. This simple model is untenable; the pelite unit overlying the marble is generally <100 m thick and includes many thin psammitic beds. It passes up into psammites (Boulder Bed) and quartzites containing very much less muscovite, whereas the total thickness of metasomatic rocks can reach 8 m. This paradox is resolved by considering the three-dimensional distribution of diopside rock and green marble. The cross-section (Fig. 1) shows that they are thickest at the crest of the main D3 antiform, thinning or vanishing on the limbs. This is a primary effect, because other beds do not show any parallel change in thickness, as would be the case if it were tectonically induced. Hence the metasomatic lithologies effectively comprise a conduit with the form of a flattened tube extending parallel to the hinge line of the D3 folds at the top of the marble sequence. If water was focused laterally into these tubes along the pelite over distances of 1–2 km, as well as vertically over at least 100 m, there is enough pelite to provide the water to infiltrate the marble and cause the observed metasomatism.

Once the water generated by pelite dehydration is focused into these channels in marble, it flushes out  $\text{CO}_2$  and equilibrates with minerals such as diopside or forsterite and calcite. Can we trace what happens to it when it finally leaves the marble? Shortly above the CMF in the Connemara stratigraphy is the Cleggan Boulder Bed, and within this broadly psammitic formation are sporadic isolated occurrences of garnet (grossular-andradite) skarn, fringed with epidote; the largest is several tens of metres across and occurs at a structural high (plunge culmination) near Maam in eastern Connemara<sup>16</sup>. Figure 3 is an activity diagram modified from Bowers *et al.*<sup>17</sup> and indicates the composition of fluid equilibrated with diopside and calcite superimposed on the stability fields for Ca–Al silicates in a quartz-saturated system. The fluid lies in the garnet field and will therefore react with plagioclase or sillimanite in a quartzofeldspathic rock to produce first epidote, then Ca garnet. We interpret these small skarns as breakout features formed where fluid that had travelled along the marble channels to higher structural levels passed back out into the overlying rocks and became dissipated.

Figure 4 is a summary of the overall flow pattern, illustrating the focusing of fluid to form first metasomatic marbles, then

FIG. 4 Schematic diagram to show fluid flow during devolatilization and metasomatism into and within the CMF, and the coupled formation of skarn at a plunge culmination. Metasomatic rocks are shown stippled and the thickness of arrows indicates relative fluxes. The scale is only to represent the order of magnitude of the process, and the skarn-forming part of the system is shown enlarged. The dashed ornament indicates the axial surfaces of D2 and D3 folds.



skarn. This episode of flow was a singular event in the history of the belt, occurring during peak-grade metamorphism. It may have been triggered by the development of porosity when dolomite and quartz reacted to produce diopside cores in hitherto impermeable, internally buffered marble, and accelerated as dolomite then reacted with infiltrating water, giving rise to further secondary porosity<sup>18,19</sup>. Even with increased porosity, the kilometre-scale focused flow along fold hinges in the CMF is possible only if there are breakout points with rapid hydraulic connection to areas of relatively low fluid pressure, so that fluid is driven into the metasomatic conduits because fluid pressure is lower than in nearby beds. In an over-pressured system, convective recharge and recirculation are then impossible<sup>3</sup>.

A final question raised by the model illustrated in Fig. 4 is whether the type of abrupt fluid loss event we propose has implications for the rheological behaviour of the fold belt. The peak-metamorphic fold structures along the central axis of Connemara where the CMF occurs are rather less tight than those to north and south. Because deformation mechanisms in high-grade metamorphism are often fluid-dependent, the loss of fluid and fluid pressure, once flow through the marble conduits began, may have caused an increase in silicate rock strength, so that regional strain became progressively partitioned into those rocks sufficiently far from the marble aquifer that they remained unaffected by the fluid loss. Because metasomatic rocks are common in high-grade marbles, the implications of such a process may be very widespread. □

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## A neurological dissociation between perceiving objects and grasping them

M. A. Goodale, A. D. Milner\*, L. S. Jakobson & D. P. Carey

Department of Psychology, University of Western Ontario, London, Canada N6A 5C2

\* Psychological Laboratory, University of St Andrews, St Andrews, Fife KY16 9JU, UK

STUDIES of the visual capacity of neurological patients have provided evidence for a dissociation between the perceptual report of a visual stimulus and the ability to direct spatially accurate movements toward that stimulus. Some patients with damage to the parietal lobe, for example, are unable to reach accurately towards visual targets that they unequivocally report seeing<sup>1,2</sup>. Conversely, some patients with extensive damage to primary visual cortex can make accurate pointing movements or saccades toward a stimulus presented in their 'blind' scotoma<sup>3–5</sup>. But in investigations of visuomotor control in patients with visual disorders, little consideration has been given to complex acts such as manual prehension. Grasping a three-dimensional object requires knowledge not only of the object's spatial location, but also of its form, orientation and size. We have examined a patient with a profound disorder in the perception of such object qualities. Our quantitative analyses demonstrate strikingly accurate guidance of hand and finger movements directed at the very objects whose qualities she fails to perceive. These data suggest that the neural substrates for the visual perception of object qualities such as shape, orientation and size are distinct from those underlying the use of those qualities in the control of manual skills.

The patient, D.F., is a 35-year-old woman who suffered irreversible brain damage following carbon monoxide intoxication. Magnetic resonance imaging revealed damage ventrally in

the lateral occipital region (mainly areas 18 and 19), and in the parasagittal occipitoparietal region, but apparently sparing most of area 17 (ref. 6). There was also some indication of localized damage in the basal ganglia. Three to six months after her accident, neuropsychological and psychophysical testing revealed the presence of a profound 'visual form agnosia'<sup>7,8</sup>. D.F. showed poor perception of shape or orientation, whether this information was conveyed by colour, intensity, stereopsis,

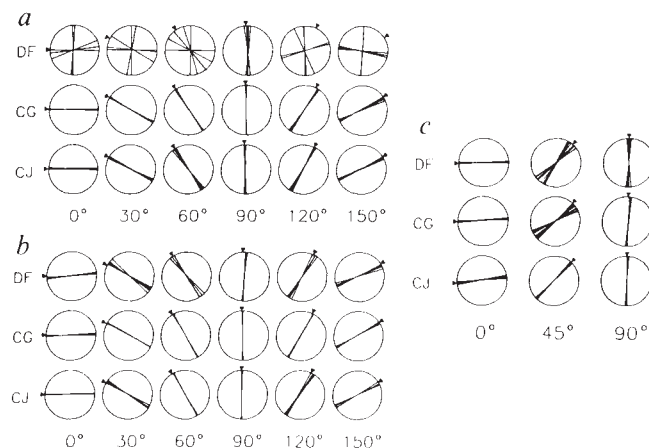
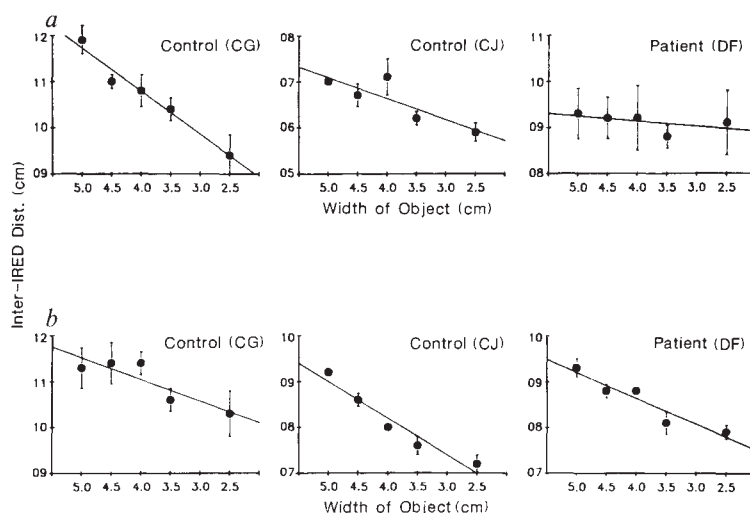


FIG. 1 The performance of the patient (D.F.) contrasted with that of two control subjects (C.G. and C.J.) of the same gender, age and handedness. Performance on each task was assessed through analysis of video records of each response. *a*, Polar plots indicate the orientation of a hand-held card which subjects were asked to match with the orientation of a slot (12.5 cm long, 3.8 cm wide, and 2 cm deep) in an upright disc, presented at a viewing distance of about 45 cm. The triangular mark on the circumference of each circle indicates the true orientation of the slot during the depicted trials. Although the patient's responses seemed to be somewhat more accurate when the slot was in the vertical (90°) orientation, each trial began with the card being held in the vertical position, which may have biased it to be the default orientation when D.F. was uncertain. *b*, Polar plots indicate the orientation of the card just before it made contact with the disc in the visually guided posting task. *c*, Polar plots indicate the orientation of a hand-held card when subjects were asked to position the card, with eyes closed, to match an imagined slot at 0°, 45° or 90°.

\* To whom correspondence should be addressed.

FIG. 2 In both the tasks described below, five white plaques (each with an overall area of 25 cm<sup>2</sup> on the top surface, but ranging in size from a 5 × 5 cm square to a rectangle measuring 2.5 × 10 cm) were presented, one at a time, at a viewing distance of approximately 45 cm. Infrared light-emitting diodes (IREDs) were attached to the tips of the index finger and thumb of the right hand. The position of each IRED was recorded by two infrared-sensitive cameras and stored on a WATSMART computer (Northern Digital Inc., Waterloo, Canada); the three-dimensional coordinates of the finger and thumb trajectories during a grasping movement and the changing distance between them were later reconstructed off-line at 100 Hz. *a*, The first task was to indicate manually the front-to-back extent of each object over a series of randomly ordered trials (means and s.e. values indicated for each object). In the patient (D.F.), unlike the controls (C.G. and C.J.), the aperture between the finger and thumb was not systematically related to the width of the target objects when performing this task ( $r(19) = +0.10$  (n.s.), versus  $r(19) = +0.82$  ( $P < 0.01$ ) and  $r(19) = +0.65$  ( $P < 0.01$ )). In interpreting these graphs it is the slope of the function that is important rather than the absolute values plotted, as the placement of the IREDs and the size of the hand and fingers varied somewhat from subject to subject. *b*, In the second task, subjects were asked to reach out and pick up each target object, again over a series of trials. In this case, the maximum aperture between the index finger and thumb, which was achieved well before contact with the object, was systematically related to the width of the objects in both the patient ( $r(19) = +0.81$



( $P < 0.01$ )) and the control subjects ( $r(18) = +0.65$  ( $P < 0.01$ ) and  $r(19) = +0.91$  ( $P < 0.01$ )). The trial-to-trial variance in D.F.'s performance was far greater on the perceptual matching task (*a*) than it was on the grasping task (*b*).

motion, proximity, continuity or similarity. Extensive visual testing indicated that the patient's visual form agnosia could not be reduced to a simple sensory deficit.

At the time of our testing, 15 months after the accident, D.F.'s verbal IQ was above average but the visual form agnosia<sup>7,8</sup> persisted. Also still present was a striking dissociation between the patient's ability to perceive object orientation and her ability to direct accurate reaching movements toward objects in different orientations<sup>8</sup>. We tested her orientation perception in several ways: to choose which of four line orientations depicted on a card matched the orientation of a large slot in an upright disk; to turn a hand-held card until its orientation matched that of the slot; and to indicate verbally the orientation of an oblong block placed on the table in front of her. Despite the variety of response options, D.F.'s performance on all these perceptual tasks was grossly impaired: her responses were highly variable and included gross errors such as judging horizontal to be vertical (see Fig. 1*a*).

In sharp contrast to her performance on these perceptual tasks, when the patient was asked to reach out and 'post' the hand-held card through the slotted disc described above, her performance was excellent (see Fig. 1*b*). Indeed, analysis of video records of each reaching movement revealed that, like the controls, D.F. began to orient the card correctly even as her hand was being raised from the start position in this task. Similarly, when asked to pick up a block placed at different orientations on the table surface in front of her, she oriented her hand appropriately very early in the reaching movement and grasped the object normally.

D.F.'s difficulty is not one of comprehension. When asked to imagine a slot at different orientations with her eyes closed, she could rotate the hand-held card to the imagined orientation just as accurately as the two age-matched controls (see Fig. 1*c*). Thus, her difficulty seems to be in using visual orientation information for perceptual or cognitive purposes, even though such information is accurately used in visuomotor action. The opposite dissociation has been described in patients with optic ataxia, who can perceive orientation well but are poor at moving their hand into an oriented slot<sup>2</sup>.

The persistence of this remarkable dissociation led us to investigate whether other aspects of object form, such as size, could modulate D.F.'s grasping responses. Accordingly we constructed five pairs of white plaques of equal area but different

dimensions, ranging from a square to an elongated rectangle<sup>9</sup>. As an initial perceptual test, the objects were presented as pairs in all possible combinations, and D.F. was asked to indicate whether the objects were alike or different. Whereas normal subjects had no difficulty discriminating any of the stimuli on this task, the patient scored no better than chance (52%). In a task of perceptual matching, D.F. and the two control subjects were asked to indicate with the index finger and thumb of their right hands the front-to-back extent (the width) of plaques placed one at a time on the table in front of them. Unlike the two control subjects, D.F.'s estimates did not change as a function of the width of the plaques (Fig. 2*a*). Thus, in both her verbal and her manual responses there was no evidence that the patient was sensitive to differences in the dimensions of the stimuli.

When we asked D.F. to reach out and pick up the plaques the results were quite different. Now, the aperture between her index finger and thumb was systematically related to the width of the object (see Fig. 2*b*), a relationship reliably observed in studies of normal prehension<sup>10</sup>. In fact, her performance was indistinguishable from that of our controls. (To confirm that she understood the perceptual matching task, we asked D.F. to indicate the width of an imagined plaque with her finger and thumb. She now showed accurate scaling with a correlation of  $+0.78$  between grip size and imagined width.)

Our data show that a person with brain damage may retain the ability to calibrate normal aiming and prehension movements with respect to the orientation and dimensions of objects, despite a profound inability to report, either verbally or manually, these same visual properties. This dissociation suggests that at some level in normal brains the visual processing underlying 'conscious' perceptual judgements must operate separately from that underlying the 'automatic' visuomotor guidance of skilled actions of the hand and limb. Note that this inferred separation does not correspond to the two streams of output from primary visual cortex generally identified in the primate brain—a ventral one for object identification and a dorsal one for spatial localization<sup>11,12</sup>. Our observations indicate separate processing systems not for different subsets of visual information, but for the different uses to which vision can be put. If correct, our reasoning suggests that in trying to understand the functions of the many different visual areas in primate cortex (as well as subcortical visual pathways) it may be



important to examine the pattern of their output connections, in addition to their visual inputs and processing characteristics. □

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## Long-term potentiation of NMDA receptor-mediated synaptic transmission in the hippocampus

Zafar I. Bashir\*, Simon Alford\*, Stephen N. Davies\*†, Andrew D. Randall‡§ & Graham L. Collingridge\*§||

Departments of \* Pharmacology and ‡ Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK and § Department of Pharmacology, The Medical School, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

**NEUROTRANSMISSION at most excitatory synapses in the brain operates through two types of glutamate receptor termed  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors; these mediate the fast and slow components of excitatory postsynaptic potentials respectively<sup>1–3</sup>. Activation of NMDA receptors can also lead to a long-lasting modification in synaptic efficiency at glutamatergic synapses; this is exemplified in the CA1 region of the hippocampus, where NMDA receptors mediate the induction of long-term potentiation (LTP)<sup>4</sup>. It is believed that in this region LTP is maintained by a specific increase in the AMPA receptor-mediated component of synaptic transmission<sup>5,6</sup>. We now report, however, that a pharmacologically isolated NMDA receptor-mediated synaptic response can undergo robust, synapse-specific LTP. This finding has implications for neuropathologies such as epilepsy and neurodegeneration, in which excessive NMDA receptor activation has been implicated<sup>7</sup>. It adds fundamentally to theories of synaptic plasticity because NMDA receptor activation may, in addition to causing increased synaptic efficiency, directly alter the plasticity of synapses.**

Synaptic responses evoked in area CA1 comprise an excitatory postsynaptic potential (e.p.s.p.), that is mediated by AMPA and NMDA receptors, and an inhibitory postsynaptic potential (i.p.s.p.), the first phase of which is mediated by GABA<sub>A</sub> receptors. To investigate whether the NMDA receptor-mediated component of the synaptic response is able to undergo LTP, we blocked the AMPA receptor-mediated component with 10  $\mu$ M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the GABA<sub>A</sub> receptor-mediated component with 50  $\mu$ M picrotoxin. Two separate inputs were stimulated alternately (each at 0.033 Hz) so as to evoke small (0.4–0.9 mV) NMDA receptor-mediated field

e.p.s.ps. After obtaining a stable baseline for about 15 min, a period of high-frequency (tetanic) stimulation was delivered (20–25 shocks at 100 Hz, test intensity) and responses were then followed for at least a further 45 min. In 6 out of 8 slices examined in this way, synapse-specific LTP (defined as at least a 20% increase in amplitude 45 min after the tetanus) was obtained (Fig. 1a, b). To ensure that the potentiated responses were mediated entirely through NMDA receptors, the specific NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (AP5; 20  $\mu$ M) was applied; this reversibly blocked the response on each occasion ( $n = 3$ ; Fig. 1b). We next investigated whether LTP of NMDA receptor-mediated responses required NMDA-receptor activation during the tetanus. We first delivered a tetanus in the presence of 50  $\mu$ M AP5, a concentration of antagonist that blocks induction of conventional LTP, and then, after washout of AP5, applied a second identical tetanus to the same input. As shown in Fig. 1c, AP5 reversibly blocked the induction of LTP of the NMDA receptor-mediated response ( $n = 3$ ).

Following the induction of LTP, the largest change in the synaptic waveform occurred several tens of milliseconds after the stimulus and was manifest as a burst of population spikes (Fig. 1a). From about 30 ms after the stimulus, a GABA<sub>B</sub> receptor-mediated i.p.s.p.<sup>8</sup> is coincident with the NMDA receptor-mediated synaptic component. The potentiation might therefore result, in part, from a long-lasting depression of GABA<sub>B</sub> receptor-mediated synaptic inhibition, rather than a direct potentiation of the NMDA receptor-mediated response. To preclude possible alterations in the GABA<sub>B</sub> receptor-mediated i.p.s.p., we applied the GABA<sub>B</sub> receptor antagonist 2-hydroxysaclofen (500  $\mu$ M). Under these conditions, in 5 out of 7 slices, tetanic stimulation resulted in LTP like that induced in the absence of 2-hydroxysaclofen.

The most pronounced change following tetanization is in the ability of the response to elicit spike firing, reflected in the appearance of asynchronous population spikes (Fig. 1a). This is reminiscent of so-called E–S potentiation (e.p.s.p. to spike coupling) and could conceivably be due to modification of voltage-dependent conductances that are evoked by the NMDA receptor-mediated e.p.s.p., rather than a direct alteration in the NMDA receptor-mediated conductance *per se*. In the next series of experiments we investigated whether LTP of the NMDA receptor-mediated synaptic current occurred, using the perforated patch method of whole-cell recording from brain slices<sup>9</sup>; this provides a high-resolution voltage-clamp of the NMDA receptor-mediated response<sup>10,11</sup> without the problem of intracellular dialysis, which reduces the likelihood of inducing LTP with time<sup>12</sup>. Using this method, in the presence of CNQX and picrotoxin, we obtained LTP in each of 7 cells tested (Fig. 2). The mean amplitude of NMDA receptor-mediated synaptic currents, recorded at a membrane potential of  $-70$  mV, 30 min following the tetanus, was  $143 \pm 13\%$  of control ( $P < 0.01$ ). In addition, we obtained similar LTP in each of two cells tetanized in two slices treated additionally with the GABA<sub>B</sub> antagonist CGP 35348 (500  $\mu$ M; Fig. 3). The potentiated synaptic current was blocked by AP5 ( $n = 2$ ) and increased in amplitude with membrane depolarization ( $n = 2$ ), confirming that it represented an NMDA receptor-mediated synaptic current in the postsynaptic cell (Fig. 3).

Our data demonstrate that NMDA receptor-mediated synaptic transmission is able to undergo a long-lasting modification. Previous reports have failed to observe significant potentiation of the isolated NMDA receptor-mediated synaptic response<sup>5,6</sup>, possibly because it is more difficult to induce LTP of the isolated NMDA receptor-mediated component (compared with conventional LTP). A possible reason for this is that low-frequency activation of the NMDA receptor system, which is minimized under physiological conditions by synaptic inhibition<sup>3,13</sup>, can reduce the probability of inducing LTP<sup>14</sup>. For this reason we used a stimulus intensity that evoked small NMDA receptor-mediated e.p.s.ps and performed the voltage-clamp experiments

† Present address: Division of Physiology, School of Biomedical Sciences, Marischal College, Aberdeen AB9 1AS, UK.

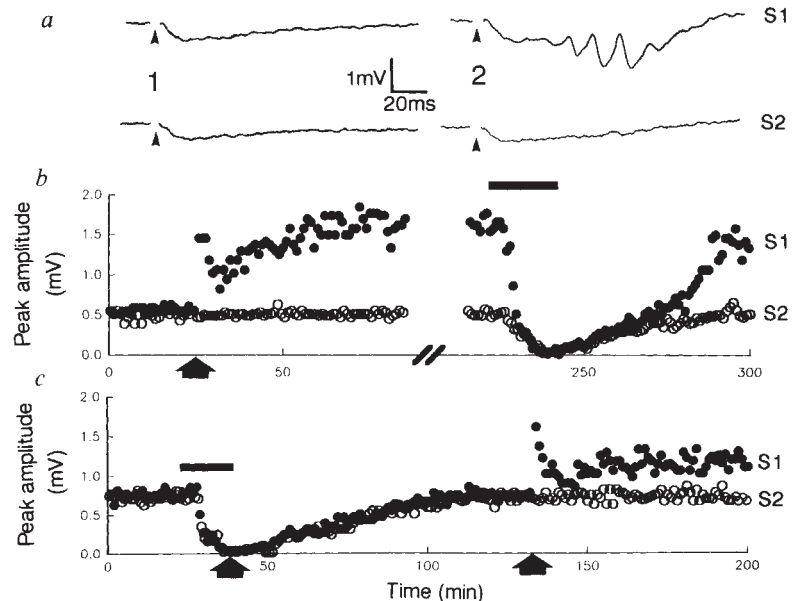
|| To whom correspondence should be addressed at the University of Birmingham.

at  $-70$  mV (a potential at which the NMDA receptor conductance is limited by its voltage-dependent block by  $Mg^{2+}$ ; refs 15, 16). Because the conditions required to isolate the NMDA receptor-mediated response might have made it harder to induce LTP<sup>14</sup>, it does not necessarily follow that under physiological conditions LTP of the NMDA receptor-mediated e.p.s.p. is a less robust phenomenon than that of conventional LTP.

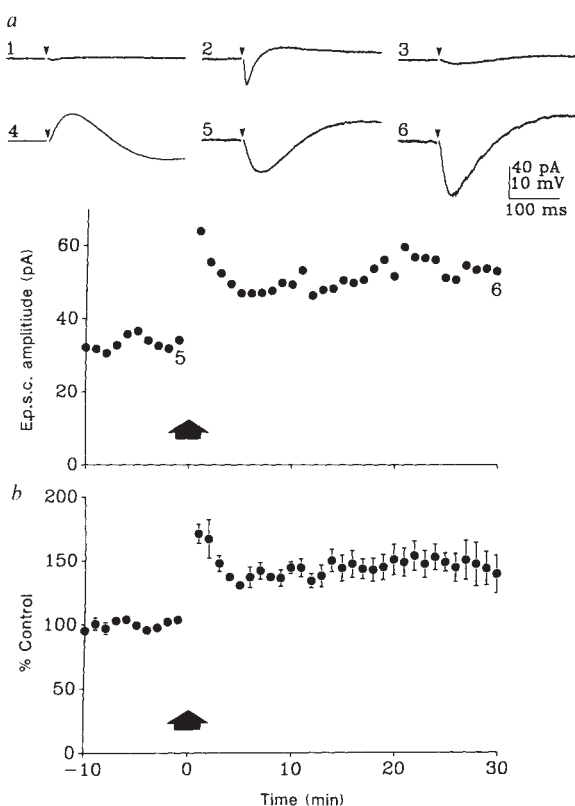
**FIG. 1** Potentiation of NMDA receptor-mediated field e.p.s.ps. Field e.p.s.ps were recorded from stratum radiatum in response to alternate stimulation of two sets of Schaffer collateral-commissural fibres (S1 and S2; each pathway stimulated every 60 s). CNQX ( $10 \mu M$ ) and picrotoxin ( $50 \mu M$ ) were applied to block the AMPA and  $GABA_A$  receptor-mediated components of synaptic transmission respectively. This virtually abolished synaptic transmission; the stimulus intensity was then increased by 33–200% to an intensity (test intensity) that evoked field e.p.s.ps of between 0.4 and 0.9 mV. **a**, A single tetanus (25 shocks, 100 Hz, test intensity) delivered to S1 resulted in rapid, robust and synapse-specific LTP. Traces are averages of 3 records obtained before and 120 min after induction of LTP. **b**, Both control and potentiated responses were reversibly blocked by bath application of  $20 \mu M$  AP5 (applied for the duration of the bar). **c**,  $50 \mu M$  AP5 (applied for the duration of the bar) blocked synaptic transmission in both inputs. A tetanus applied at this time did not induce LTP, whereas an identical tetanus delivered to the same input, after washout of AP5, induced synapse-specific LTP. Graphs plot peak amplitude of individual synaptic responses versus time. Because following LTP the peak response involves a population spike, the magnitude of the potentiation does not directly represent the size of the underlying synaptic potentiation. Arrows mark the time of tetanization (25 shocks, 100 Hz, test intensity). In this and subsequent traces arrowheads indicate the position of blanked stimulus artefacts.

**METHODS.** Rat hippocampal slices ( $400 \mu m$  thick) were prepared using standard techniques. The CA3 regions were discarded and slices were perfused with medium containing: (mM) NaCl, 124;  $NaHCO_3$ , 26; KCl, 3;

Under experimental conditions in which both NMDA and AMPA receptors mediate synaptic responses, LTP is reported to involve a specific enhancement of the AMPA receptor component in the hippocampus<sup>5,6</sup> (but see refs 17 and 18 for LTP in the neocortex). This has been used to argue that LTP in the hippocampus is maintained solely by postsynaptic mechanisms<sup>5,6</sup>. Although we have not addressed, and therefore cannot

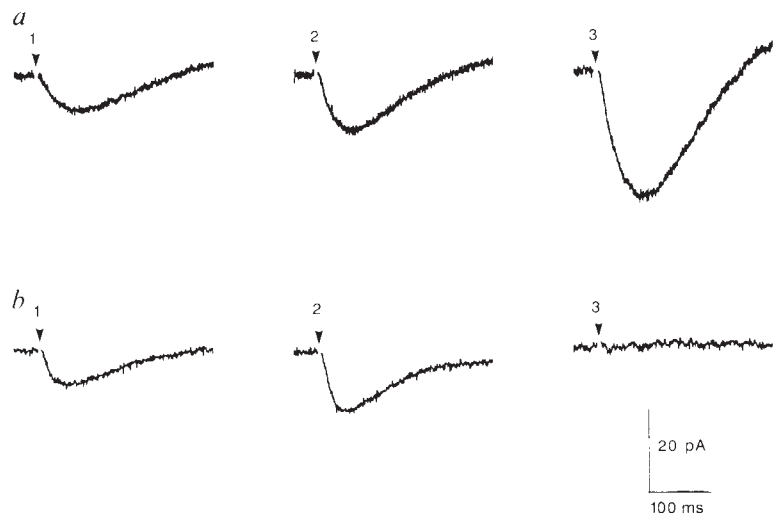


$NaH_2PO_4$ , 1.25;  $CaCl_2$ , 2;  $MgSO_4$ , 1; D-glucose, 10 (bubbled with 95%  $O_2$ /5%  $CO_2$ ). The Schaffer collateral-commissural pathway was stimulated with bipolar electrodes placed in stratum radiatum. Data are presented as means  $\pm$  s.e.m. and probabilities were estimated using Student's paired *t*-tests.



**FIG. 2** LTP of NMDA receptor-mediated synaptic currents recorded with the perforated whole-cell patch technique. Cell-attached patch-clamp recordings were made using the 'blind' patch technique<sup>30</sup> with electrodes containing (mM): caesium methanesulphonate, 130; NaCl, 5;  $CaCl_2$ , 1; HEPES, 5; EGTA, 10, pH 7.3. Nystatin ( $100 \mu g ml^{-1}$ ) was added to the electrode solution immediately before the experiment. Nystatin causes the formation of ion-permeant pores in the patch, enabling recordings to be made in the whole-cell mode without dialysis of the cell contents<sup>31</sup>. Seal resistances measured immediately after achieving cell-attached recordings ranged from 1 to 8 G $\Omega$ . The formation of the perforated whole-cell patch recording was monitored by development of whole-cell capacitance transients and emergence of synaptic currents. **a**, Immediately after attachment of the pipette to the cell membrane, the synaptic currents were small (1), but increased in size and stabilized after 30 min (2). Addition of  $10 \mu M$  CNQX and  $50 \mu M$  picrotoxin to the bathing medium left an NMDA receptor-mediated inward current followed by a  $GABA_B$  receptor-mediated outward current (3). The stimulus intensity was then increased to elicit a larger synaptic current (5). (4 shows the equivalent synaptic potential recorded in current-clamp.) The potentiated synaptic current, 30 min after tetanus (20 shocks at 100 Hz, test intensity) is shown (6). The graph plots the peak amplitudes of the inward current before and following the tetanus for this cell. **b**, Graph showing normalized data from all 7 neurons (from 7 slices) from which such recordings were made, under these conditions. The mean values immediately before and 30 min following the tetanus were  $16 \pm 4$  and  $24 \pm 7$  pA, respectively. In all experiments, stimuli were delivered at 15-s intervals and the holding potential was  $-70$  mV throughout (including during the tetanus). All traces and data points are averages of 4 successive records. Mean input resistance was  $270 \pm 18 M\Omega$  and resting membrane potential was  $-52 \pm 2$  mV.

FIG. 3 LTP of NMDA receptor-mediated synaptic currents. *a*, This cell was recorded as described in Fig. 2. Responses were obtained (at  $-70$  mV) before (1) and 30 min following (2) the induction of LTP. The cell was then depolarized to  $-50$  mV (3). *b*, This cell was recorded as described in Fig. 2, except that the perfusate also contained  $500 \mu\text{M}$  CGP 35348 to block the  $\text{GABA}_\text{B}$  receptor-mediated i.p.s.c. Responses were obtained (at  $-70$  mV) before (1) and 30 min following (2) the induction of LTP. The potentiated synaptic current was blocked by  $40 \mu\text{M}$  AP5 (3).



exclude, differential expression of the two synaptic components during LTP, our positive finding that LTP of the NMDA receptor-mediated response can occur refutes the argument that presynaptic mechanisms are not involved in the maintenance of LTP. Indeed, other lines of evidence strongly suggest that presynaptic mechanisms contribute to the expression of LTP in the hippocampus<sup>12,19-22</sup>. With respect to LTP of the NMDA receptor component, this could be mediated by a persistent presynaptic change and/or a postsynaptic alteration, such as modification of the NMDA receptor channel complex. It may be relevant that activation of protein kinase C can upregulate NMDA currents<sup>23</sup>.

As NMDA receptor gated channels are permeable to  $\text{Ca}^{2+}$  (refs 24-26), a consequence of LTP of this component is that a given synaptic input should provide increased  $\text{Ca}^{2+}$  entry by this route. This is likely to have implications for both physiological and pathological function. In terms of LTP, it means that a tetanus, in addition to increasing the efficiency of synaptic transmission, could directly alter the plasticity in the pathway for long periods. Subsequent tetani could result in correspondingly greater enhancements of synaptic transmission, leading to the genesis of epileptiform activity<sup>27-28</sup> and, taken to extreme, cell death<sup>29</sup>. □

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## Polarized sorting of glypiated proteins in hippocampal neurons

Carlos G. Dotti, Robert G. Parton & Kai Simons

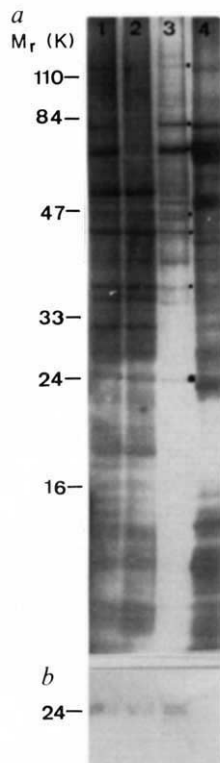
Cell Biology Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg, Germany

OUR recent studies suggested that neurons and epithelial cells sort viral glycoproteins in a similar manner. The apical influenza virus haemagglutinin was preferentially delivered to the axon of hippocampal neurons in culture, whereas the basolateral vesicular stomatitis virus glycoprotein was sorted to the dendrites.<sup>1</sup> To investigate whether other membrane proteins showed similar sorting in neurons and epithelial cells, we have analysed the localization of a glypiated (glycosylphosphatidylinositol-anchored) protein, Thy-1, in hippocampal neurons in culture. In MDCK and other epithelial cells, endogenous glycosylphosphatidylinositol (GPI)-anchored proteins, as well as mutated exogenous proteins containing the GPI-attachment signal, undergo preferential delivery to the apical surface<sup>2-4</sup>. This polarized sorting of GPI-anchored proteins has been proposed to occur by the same mechanisms as the sorting of glycolipids to the apical surface<sup>5,6</sup>. We report here that the neuronal GPI-protein Thy-1 is present in hippocampal neurons in culture and is exclusively located on the axonal surface. This finding further strengthens our hypothesis that the mechanisms of sorting of surface components may be similar in neurons and epithelial cells.

In a first series of experiments we investigated whether Thy-1 existed as a GPI-anchored protein on the surface of hippocampal cells in culture. To do this we took advantage of the fact that on treatment of the detergent phase of extracted cells with phosphatidylinositol-specific phospholipase C (PI-PLC), GPI-anchored proteins undergo a conversion from a hydrophobic to a hydrophilic state, thus partitioning into the aqueous phase<sup>2,7</sup>.

The detergent phase of Triton X-114-extracted stage 5 hippocampal cells<sup>8</sup> was incubated with PI-PLC and the shift of proteins from the detergent into the aqueous phase was monitored by polyacrylamide gel electrophoresis (Fig. 1). After PI-PLC treatment several proteins present in the detergent fraction





appeared in the aqueous phase (dots). One of these proteins (asterisk) had the relative molecular mass expected for Thy-1 (ref. 7). This was confirmed by western blot analysis (Fig. 1b). Thus, Thy-1 showed the expected behaviour for a GPI-anchored protein.

Next we examined the distribution of Thy-1 immunoreactivity on the surface of hippocampal neurons in culture using light and electron microscopy. Figure 2 illustrates the localization of the Thy-1 protein by immunofluorescence in hippocampal neurons after 14 days in culture. At this time, all the morphological and molecular characteristics of axons and dendrites have been established and numerous synaptic contacts have been created<sup>9</sup>. An anti-MAP-2 antibody was also used to distinguish the axons from the dendrites<sup>10</sup>. Thy-1 immunoreactivity was detected along virtually all axons in a discontinuous and

FIG. 1 *a*, Identification of Thy-1 as a GPI-anchored protein in cultured hippocampal cells. Lane 1, detergent phase of hippocampal cell extracts before treatment with *Staphylococcus aureus* phospholipase C (PI-PLC); lane 2, same detergent phase after treatment with PI-PLC; lane 3, aqueous phase after treatment of the detergent phase with PI-PLC; lane 4, proteins partitioning in the starting aqueous phase. Several major bands appear in the aqueous phase after PI-PLC treatment (dots between lanes 3 and 4). One shows the molecular weight expected for Thy-1 (asterisk). In the corresponding western blots (*b*) of lanes 1, 2, 3 and 4, a mouse monoclonal anti-Thy-1 antibody was used to show that the major band of relative molecular mass 24,000 (24K) seen in the upper panel (asterisk) corresponded to Thy-1. Thy-1 is not present in the starting aqueous phase. Repeated treatment with PI-PLC of the detergent phase (lane 2) removed all the Thy-1 into the aqueous phase.

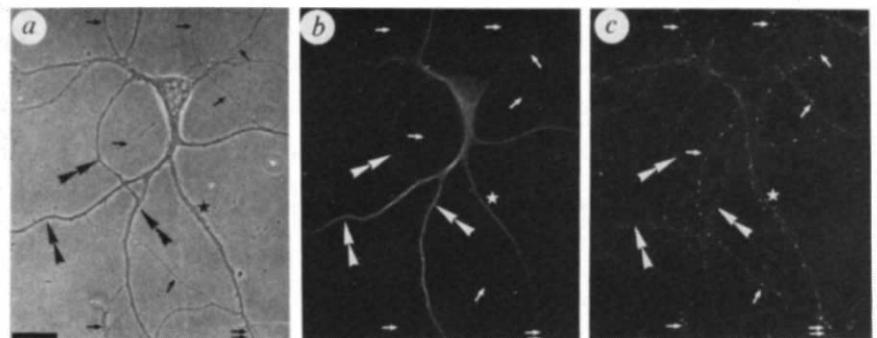
METHODS. The protocol was adapted from Lisanti *et al.*<sup>2</sup>. Hippocampal neurons ( $5 \times 10^6$ ) maintained for 14 days in culture were extracted in TBS (10 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM EDTA) containing 1% Triton X-114 for 1 h at 4 °C. Cell extracts were clarified by centrifugation and the resulting supernatant subjected to temperature-dependent phase separation. Aliquots were taken from the detergent (lane 1) and aqueous phase (lane 4) and the detergent phase was re-extracted in 10 volumes of TBS containing 0.06% Triton X-114. The re-extracted detergent phase was then diluted in 100 mM Tris, 50 mM NaCl, 1 mM EDTA plus 6 units  $\text{ml}^{-1}$  of PI-PLC (Boehringer) for 1 h at 37 °C. After PI-PLC treatment, the sample was diluted in TBS containing 2% Triton X-114 and subjected to temperature separation. The detergent phase was kept (lane 2) and the aqueous phase was re-extracted in TBS plus 10% Triton X-114. The aqueous phase (lane 3) was analysed. In all steps, the solutions contained a mixture of anti-proteases<sup>16</sup>. Proteins in the different detergent and aqueous phases were concentrated in trichloroacetic acid, precipitated, and solubilized in Laemmli sample buffer. PAGE (10%) were run according to Laemmli<sup>17</sup>. Relative molecular mass markers (prestained, Bio-Rad), are indicated in K. Proteins were detected by the silver staining technique. Western blots were performed according to Towbin *et al.*<sup>18</sup>. Thy-1 immunoreactivity was assessed using a monoclonal antibody which recognizes the Thy-1.1 determinant (clone MRC-OX 7)<sup>19</sup>.

dotted pattern. Quantitation showed that 80% of 287 axons were Thy-1 positive. In addition to the axonal labelling, clusters of Thy-1 protein could also be detected along some dendrites and cell bodies. The dendritic labelling appeared anomalous for two reasons, however. First, labelling was not observed on all dendrites (50% of the MAP-2-positive primary dendrites of 500 cells had Thy-1 immunoreactivity) and second, the labelling did not seem to follow the trajectory of the dendrite but could be often seen at sites near, but not along, the dendritic surface. Furthermore, the Thy-1 labelling could always be seen beyond the end of the dendrites, suggesting that Thy-1 protein was in axons running along the dendritic surface. The few glial cells present in this culture appeared unlabelled.

The preferential localization of Thy-1 along axons was unequivocally confirmed in paraformaldehyde-fixed cells

FIG. 2 Comparison of Thy-1 immunoreactivity in axons and dendrites of hippocampal neurons in culture. The phase contrast photograph (*a*) shows a typical hippocampal pyramidal cell after 14 days in culture. The cell dendrites as well as numerous axons from neighbour cells can be distinguished morphologically; dendrites are thick and taper, whereas the axons are thin and of uniform diameter. Immunostaining with an anti-MAP-2 antibody labels the cell dendrites (*b*). Thy-1 immunoreactivity (*c*), using a rabbit  $\text{F(ab')}_2$  fragment<sup>20</sup>, is present in the axons in the form of clusters (arrows). Some of the cell dendrites (double arrowheads) are devoid of Thy-1 immunoreactivity, whereas in others the labelling is unequivocal (asterisk). Notice, however, that even in this dendrite the Thy-1 labelling can be seen beyond the dendritic end (double arrows). A similar pattern of Thy-1 immunoreactivity was found using the monoclonal antibody MRC-OX7 (not shown). Bar, 10  $\mu\text{m}$ .

METHODS. Hippocampal cells were obtained from trypsinized hippocampus from 18-day rat embryos and then plated onto polylysine-coated glass coverslips and maintained in serum-free medium as described previously<sup>11</sup>. Proliferation of non-neuronal cells was prevented by cytosine arabinoside. After 14 days in culture, cells were incubated with a  $\text{F(ab')}_2$  rabbit anti-Thy-1



antibody diluted in culture medium and left at 37 °C for 30 min. Immediately after incubation, coverslips were briefly rinsed in Hank's Balanced Salt Solution and fixed in 4% paraformaldehyde for 20 min, washed in PBS and incubated with fluorescein-conjugated anti-rabbit IgG. Subsequently, the cells were extensively washed in PBS, permeabilized with Triton X-100 (0.125% in PBS, 10 min), and incubated with a monoclonal anti-MAP-2 antibody (clone AP14) followed by a secondary anti-mouse IgG conjugated to rhodamine.

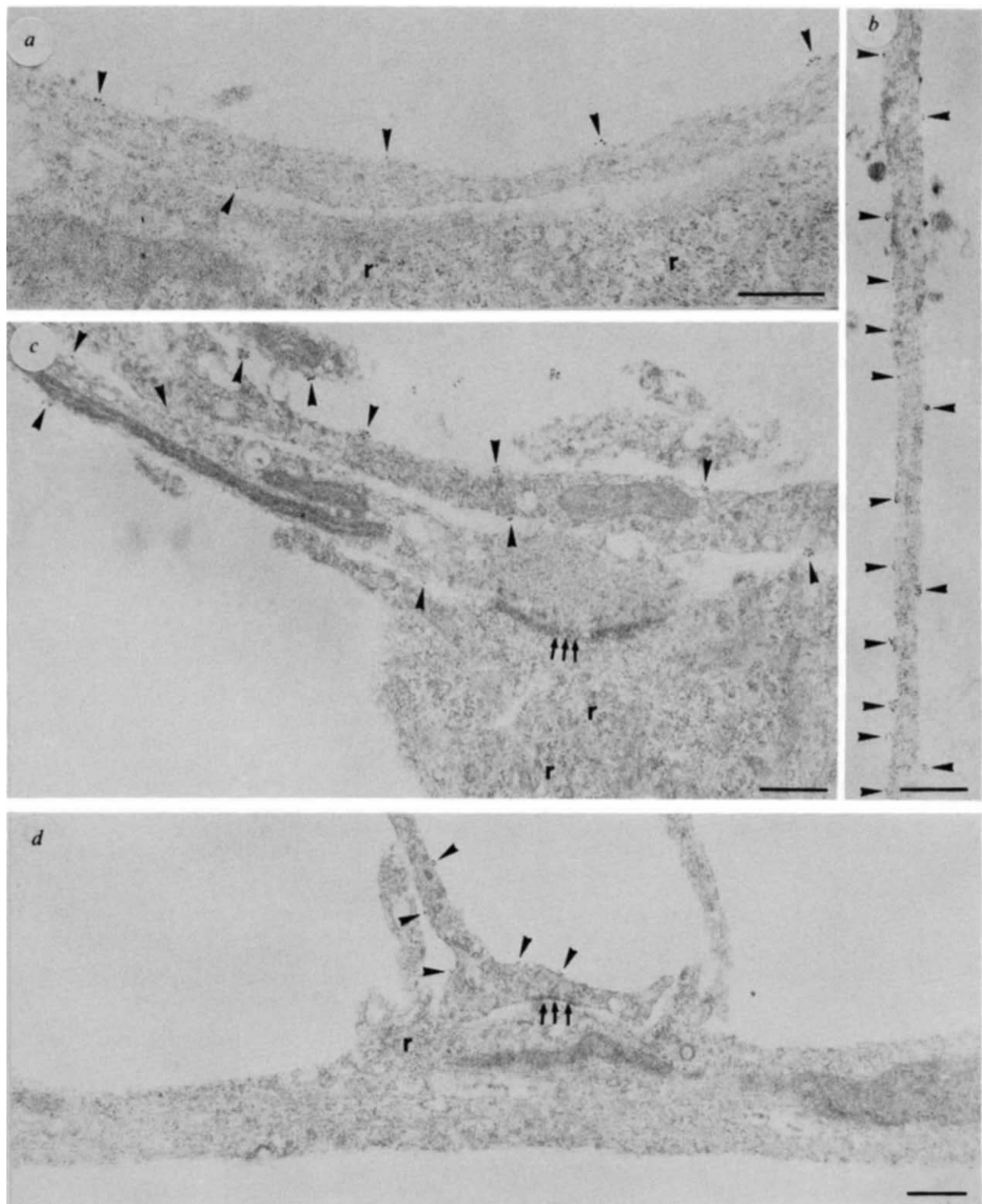


FIG. 3 Immunoelectron microscopic localization of Thy-1 in hippocampal cells in culture. *a*, Axon (thin and of uniform diameter) running along the surface of a neuronal soma. Ribosomes are located in the cell body (*r*). Clusters of Thy-1 are seen exclusively along the axonal surface (arrowheads) on both sides of the process. No Thy-1 labelling is seen on the cell body surface. *b*, Cluster of Thy-1 (arrowheads) along an isolated axon. *c*, Two axons running parallel to a dendrite, with one of them establishing a synaptic contact (arrows). Thy-1 clusters are restricted to the axonal surfaces. *d*, Axodendritic contact. Notice the lack of Thy-1 immunoreactivity along the thick dendrite (ribosomes (*r*) and postsynaptic thickening (arrows)) whereas the thin axon shows numerous gold particles (arrowheads). Bars, 0.5  $\mu$ m. METHODS. Hippocampal cells grown on polylysine-coated plastic dishes for 14 days were fixed in 8% paraformaldehyde in 250 mM HEPES, pH 7.35.

This primary fixation was required to maintain antigenicity but resulted in suboptimal morphology. After washing in HEPES buffer, the cells were incubated in PBS containing 10% FCS (Gibco) and 0.12% glycine for 20 min at room temperature. The cells were then sequentially incubated with the following reagents diluted in PBS containing 5% FCS, each for 30 min at room temperature; anti-Thy-1 monoclonal antibody, clone MRC-OX7; rabbit anti-mouse IgG (Cappel); protein A-gold 9 nm. After each step the cells were washed 5 $\times$  over 30 min with PBS. The cells were then post-fixed in 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.35, and processed for Epon embedding. After polymerization, the cells were removed from the culture dish and selected groups of cells were sectioned in the plane of the culture dish.



processed for immunoelectron microscopy (Fig. 3). Two ultra-structural parameters were used to distinguish axons from dendrites and cell bodies: synaptic vesicles and ribosomal clusters (polysomes). Whereas synaptic vesicles are predominantly in axons, polysomes are located exclusively in the cell body and dendrites, being totally absent from axons<sup>11</sup>. Using these criteria, Thy-1-positive immunoreaction appeared exclusively along axons. All dendrites were virtually devoid of reaction product. In the cases where axons were running along dendrites forming synapses *en passant*<sup>12</sup>, only the axon showed positive Thy-1 labelling which was seen on the contacting side as well as on the free surface (Fig. 3a, c). Positive Thy-1 immunoreactivity was also seen on axons which were not in contact with other processes (Fig. 3b). Thy-1 labelling was very seldom found along the cell body plasma membrane.

These observations may have important implications for the possible mechanisms of membrane biogenesis in neurons. Our finding that Thy-1 is located in the axonal membrane adds new evidence to that supporting the hypothesis that the machinery for the sorting of axonal membrane proteins in neurons may be equivalent to the apical route of MDCK cells<sup>1</sup>. Thus, different types of membrane proteins, a glycoprotein which is inserted into the membrane via a hydrophobic domain (HA of influenza virus) and a GPI-anchored glycoprotein, undergo similar polarized delivery to the axons and to the apical surfaces of neurons and MDCK cells, respectively. The sorting of the Thy-1 molecule also resembled the sorting of fowl plague virus-HA in that Thy-1 was uniformly distributed on the surface of all processes of immature (stage 3) cells (not shown).

How similar are the mechanisms operating to create the characteristic membrane composition of axons and dendrites of neurons and the apical and basolateral membrane of MDCK cells? Are glycosphingolipids sorted preferentially to axons in neurons as they are to the apical membrane in epithelial cells<sup>13</sup>? Is the axonal sorting of GPI-anchored proteins a consequence of glycolipid sorting? A further interesting question is whether there is in neurons a physical barrier, like the tight junctions in MDCK cells which prevents the mixing of membrane proteins from the different domains<sup>14</sup>. We observed Thy-1 in axons but little, if any, Thy-1 immunoreactivity in the neuronal cell bodies and practically none in the dendrites. As Thy-1 is a protein that can move in the lateral plane of the membrane<sup>15</sup>, the presence of some kind of barrier may have prevented the protein from moving from the axon into the cell body. Is this putative barrier located at the axonal hillock region?

Our study raises more questions than it answers. But the data suggest that a comparison of epithelial and neuronal cells will provide rewarding avenues for future research. □

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## Familial combined hyperlipidaemia linked to the apolipoprotein AI-CIII-AIV gene cluster on chromosome 11q23–q24

Andrew P. Wojciechowski\*, Martin Farrell\*, Paul Cullen\*, Teresa M. E. Wilson\*, Jayne D. Bayliss\*, Bernadette Farren\*, Bruce A. Griffin†, Muriel J. Caslake†, Christopher J. Packard†, James Shepherd†, Rajesh Thakker\* & James Scott\*‡

\* Division of Molecular Medicine, MRC Clinical Research Centre, Northwick Park Hospital, Watford Road, Harrow, Middlesex HA1 3UJ, UK

† Institute of Pathological Biochemistry, Glasgow Royal Infirmary, Glasgow G4 0SF, UK

FAMILIAL combined hyperlipidaemia (FCHL) is a common inherited disorder of lipid metabolism with a prevalence of 0.5–2.0% (refs 1, 2). It is estimated to cause 10% of premature coronary heart disease<sup>1,3</sup>. The underlying metabolic and genetic defects in FCHL have not been identified, but a population study has suggested an association between FCHL and an *XmnI* restriction fragment length polymorphism (RFLP) within the apolipoprotein AI-CIII-AIV gene cluster<sup>4</sup>. Here we confirm this association and show that it results from linkage disequilibrium between FCHL and the 6.6-kilobase (kb) allele of the *XmnI* RFLP. Subsequent analysis in seven FCHL families, ascertained through a proband carrying the 6.6 kb *XmnI* allele, demonstrated linkage to the AI-CIII-AIV cluster on 11q23–q24,  $\chi^2 = 6.86$  with no recombinants. This assignment will facilitate the identification of the mutation that causes hyperlipidaemia in these families.

FCHL was first described in 1973 (refs 2, 5, 6), and Goldstein *et al.* proposed a dominant mode of inheritance for the disease, with reduced penetrance in children<sup>2</sup>. About one-fifth of FCHL patients develop premature coronary heart disease<sup>1,3</sup>. The condition is characterized by an elevation of both cholesterol and triglyceride levels, although members of the same family may have elevation of either lipid alone<sup>1</sup>. Patients also have 'small dense LDL', that is, low-density lipoprotein with a relative excess of small diameter subfractions of increased density. This feature distinguishes FCHL from familial hypercholesterolaemia, which is caused by a deficiency of the LDL receptor<sup>7</sup>. Lipoprotein kinetic studies have indicated that high production rates of very low density lipoprotein (VLDL) apolipoprotein B (apo-B) from the liver or abnormal 'metabolic channelling' in the circulation may underlie FCHL<sup>1</sup>.

A study of the frequency of RFLPs in the apo-AI-CIII-AIV gene cluster has suggested an association between an *XmnI* RFLP and FCHL<sup>4</sup>. This RFLP is located approximately 2.5 kb upstream of the apo-AI gene. It is detected by an apo-AI complementary DNA probe which reveals two fragments of 8.3 kb (allele X1) and 6.6 kb (allele X2)<sup>8</sup>. To investigate this population-based association further, we genotyped 16 unrelated FCHL probands and 40 normolipidaemic controls (Table 1a).

Proband of northern European origin were selected from the lipid clinic at Northwick Park Hospital as having total serum cholesterol and triglyceride levels above the ninety-fifth percentile. In the absence of extensive published British percentile data, all lipid levels in the present study were corrected for age and sex using the percentile levels of the Lipid Research Clinics<sup>9</sup>. Mean lipid levels in this study were, however, very similar to those found in a population study in the Harrow area by our group<sup>10</sup>. At least one other first degree relative had a cholesterol

‡ To whom correspondence should be addressed.



TABLE 2 Lipid results in all family members included in linkage analysis (all values in mmol<sup>-1</sup>)

| Pedigree no. | Individual no. | Age | Sex | Total chol. | Total trig. | HDL chol. | LDL chol. | Pedigree no. | Individual no. | Age | Sex | Total chol. | Total trig. | HDL chol. | LDL chol. |
|--------------|----------------|-----|-----|-------------|-------------|-----------|-----------|--------------|----------------|-----|-----|-------------|-------------|-----------|-----------|
| 1            | II, 1          | 63  | F   | 9.4         | 5.90        | 1.09      | —         | 4            | II, 2          | 66  | M   | 5.4         | 1.06        | 1.02      | 3.92      |
| 1            | II, 2          | 51  | F   | 5.7         | 1.20        | 1.15      | 4.03      | 4            | III, 1         | 42  | M   | 7.5         | 1.75        | 1.20      | 5.54      |
| 1            | II, 3*         | 50  | M   | 9.0         | 12.00       | 0.60      | —         | 5            | I, 1           | 71  | F   | 8.8         | 7.56        | 1.09      | —         |
| 1            | III, 1         | 21  | F   | 4.9         | 0.79        | 0.85      | 3.70      | 5            | I, 2           | 84  | M   | 5.1         | 1.51        | 0.98      | 3.46      |
| 1            | III, 2         | 28  | M   | 7.1         | 3.75        | 1.06      | 4.41      | 5            | II, 1          | 44  | F   | 5.6         | 0.94        | 1.56      | 3.63      |
| 1            | III, 3         | 27  | M   | 4.1         | 0.70        | 1.02      | 2.77      | 5            | II, 2          | 45  | M   | 7.2         | 2.92        | 0.97      | 4.96      |
| 2            | II, 1          | 76  | M   | 6.1         | 1.35        | 1.54      | 3.97      | 5            | II, 3*         | 46  | M   | 9.0         | 8.28        | 1.66      | —         |
| 2            | II, 2*         | 70  | F   | 10.7        | 3.70        | 1.28      | 7.80      | 5            | II, 4          | 39  | M   | 7.4         | 2.47        | 1.22      | 5.10      |
| 2            | II, 3          | 65  | M   | 5.7         | 1.50        | 1.28      | 3.77      | 5            | III, 1         | 17  | F   | 4.1         | 0.85        | 1.27      | 2.46      |
| 2            | II, 4          | 37  | F   | 8.1         | 1.50        | 1.69      | 5.75      | 5            | III, 2         | 16  | F   | 5.0         | 1.80        | 1.53      | 2.68      |
| 2            | III, 1         | 50  | M   | 7.3         | 1.61        | 1.52      | 5.08      | 6            | II, 1          | 54  | F   | 6.1         | 0.91        | 1.91      | 3.79      |
| 2            | III, 2         | 49  | F   | 4.4         | 0.90        | 1.89      | 2.12      | 6            | II, 2          | 59  | M   | 7.3         | 1.56        | 0.93      | 5.69      |
| 2            | III, 3         | 24  | F   | 4.8         | 1.21        | 1.73      | 2.54      | 6            | II, 3*         | 54  | M   | 7.5         | 4.71        | 1.14      | 4.31      |
| 2            | III, 4         | 29  | F   | 4.9         | 0.85        | 1.19      | 3.34      | 6            | III, 1         | 28  | M   | 6.9         | 1.80        | 1.14      | 4.98      |
| 3            | II, 1          | 69  | M   | 4.6         | 0.91        | 1.05      | 3.15      | 6            | III, 2         | 20  | F   | 5.9         | 2.53        | 1.55      | 3.25      |
| 3            | II, 2          | 49  | F   | 5.2         | 0.92        | 1.48      | 3.32      | 7            | II, 1          | 55  | M   | 9.7         | 3.29        | 1.04      | 7.23      |
| 3            | II, 3          | 61  | M   | 9.3         | 3.56        | 1.30      | 6.45      | 7            | II, 2*         | 52  | M   | 7.5         | 3.40        | 1.16      | 4.86      |
| 3            | II, 4          | 47  | F   | 7.2         | 1.99        | 1.16      | 5.17      | 7            | II, 3          | 47  | F   | 4.5         | 0.80        | 1.23      | 2.92      |
| 3            | II, 5*         | 54  | M   | 7.9         | 2.32        | 1.25      | 5.63      | 7            | II, 5          | 58  | F   | 7.1         | 6.90        | 1.27      | —         |
| 3            | II, 6          | 50  | F   | 5.5         | 1.34        | 1.20      | 3.72      | 7            | II, 6          | 59  | F   | 9.4         | 4.44        | 1.27      | 6.19      |
| 3            | II, 7          | 61  | M   | 7.3         | 2.00        | 1.33      | 5.10      | 7            | III, 1         | 27  | M   | 6.9         | 3.86        | 1.13      | 4.09      |
| 3            | III, 1         | 28  | F   | 8.1         | 0.95        | 1.41      | 6.27      | 7            | III, 2         | 28  | F   | 4.8         | 0.65        | 1.60      | 2.90      |
| 3            | III, 2         | 28  | F   | 6.1         | 1.51        | 1.71      | 3.73      | 7            | III, 3         | 22  | M   | 4.3         | 0.96        | 1.39      | 2.49      |
| 3            | III, 3         | 25  | F   | 4.5         | 0.40        | 1.23      | 3.09      | 7            | III, 4         | 32  | M   | 4.6         | 0.63        | 1.22      | 3.10      |
| 4            | II, 1*         | 63  | F   | 8.9         | 3.75        | 1.08      | 6.19      | 7            | III, 5         | 26  | M   | 4.2         | 1.56        | 1.34      | 2.18      |

Lipid parameters were measured by previously published methods<sup>13</sup>. Total cholesterol (chol.) total triglyceride (trig.), HDL cholesterol and LDL cholesterol levels are in mmol<sup>-1</sup> (to convert cholesterol from mmol l<sup>-1</sup> to mg dl<sup>-1</sup>, multiply by 38.7; to convert triglyceride from mmol l<sup>-1</sup> to mg dl<sup>-1</sup>, multiply by 88.5). LDL cholesterol was calculated using the Friedewald formula<sup>24</sup>. The presence of small dense LDL was demonstrated in the proband of pedigrees 1, 2, 4 and 6 and in one first-degree relative of the proband in pedigrees 3, 5 and 7 (denoted by \*). The complete data on all the pedigrees analysed are the subject of a manuscript in preparation.

TABLE 1 Genotype frequencies and statistical analysis

(a) Comparison of apo-AI *XmnI* genotype frequencies in normolipidaemic controls and FCHL probands.

| Apo-AI <i>XmnI</i> polymorphism | Normolipidaemic |       |                      |       | FCHL     |       |                      |       |
|---------------------------------|-----------------|-------|----------------------|-------|----------|-------|----------------------|-------|
|                                 | NPH             |       | Hayden <i>et al.</i> |       | NPH      |       | Hayden <i>et al.</i> |       |
|                                 | <i>n</i>        | %     | <i>n</i>             | %     | <i>n</i> | %     | <i>n</i>             | %     |
| X1X1                            | 33              | 82.5  | 31                   | 81.6  | 8        | 50.0  | 14                   | 58.3  |
| X1X2                            | 7               | 17.5  | 6                    | 15.8  | 7        | 43.7  | 10                   | 41.7  |
| X2X2                            | 0               | 0.0   | 1                    | 2.7   | 1        | 6.3   | 0                    | 0.0   |
| Total                           | 40              | 100.0 | 38                   | 100.0 | 16       | 100.0 | 24                   | 100.0 |

(b) Chi-squared analysis

| Source of association               | (d.f.) | NPH      |          | Hayden <i>et al.</i> |          | Combined |          |
|-------------------------------------|--------|----------|----------|----------------------|----------|----------|----------|
|                                     |        | $\chi^2$ | <i>P</i> | $\chi^2$             | <i>P</i> | $\chi^2$ | <i>P</i> |
| Allelic (linkage disequilibrium)    | (1)    | 6.29     | (0.012)  | 3.34                 | (0.07)   | 9.29     | (0.002)  |
| Other sources (including epistasis) | (3)    | 0.83     | (0.84)   | 2.45                 | (0.48)   | 0.20     | (0.98)   |
| All sources                         | (4)    | 7.13     | (0.13)   | 5.79                 | (0.22)   | 9.49     | (0.05)   |

The total  $\chi^2$  for association between genotypes at the apo-AI and FCHL loci was partitioned into two components: that attributed to association between alleles (which includes linkage disequilibrium); and all other sources of genotypic association (which includes epistasis)<sup>12</sup>. The quoted *P* values (in parentheses) may be unreliable as a consequence of small expected cell probabilities and should be interpreted cautiously. d.f., Degrees of freedom; NPH, data from this study; Hayden *et al.*, data from the original population-based association study by Hayden *et al.*<sup>4</sup>; Combined, analysis of combined data from both studies.

TABLE 3 Lod score for FCHL versus the apo-AI *XmnI* RFLP and the apo-CIII microsatellite tandem repeat

| $\theta$   | 0.00  | 0.01  | 0.05  | 0.10  | 0.15  | 0.20  | 0.30  | 0.40  |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Pedigree 1 | 1.06  | 1.04  | 0.95  | 0.85  | 0.73  | 0.61  | 0.36  | 0.13  |
| Pedigree 2 | 1.46  | 1.44  | 1.33  | 1.19  | 1.05  | 0.91  | 0.60  | 0.27  |
| Pedigree 3 | -0.19 | -0.20 | -0.22 | -0.24 | -0.23 | -0.21 | -0.14 | -0.06 |
| Pedigree 4 | 0.21  | 0.21  | 0.19  | 0.17  | 0.16  | 0.14  | 0.09  | 0.05  |
| Pedigree 5 | 1.38  | 1.36  | 1.27  | 1.16  | 1.04  | 0.91  | 0.64  | 0.33  |
| Pedigree 6 | 0.86  | 0.85  | 0.78  | 0.70  | 0.61  | 0.53  | 0.35  | 0.16  |
| Pedigree 7 | 2.05  | 2.02  | 1.87  | 1.68  | 1.47  | 1.26  | 0.83  | 0.39  |
| Totals     | 6.86  | 6.72  | 6.20  | 5.39  | 4.85  | 4.16  | 2.73  | 1.27  |

The lod scores for both sexes were computed together ( $\theta_{\text{male}} = \theta_{\text{female}}$ ). Assuming a population prevalence of 1%, the frequency of the FCHL allele is 0.005. A 2% phenocopy rate was allowed for individuals with combined hyperlipidaemia and a 5% phenocopy rate was allowed for individuals with a solitary elevation of either cholesterol or triglyceride; 99% penetrance of the FCHL allele was assumed. Haplotype frequencies allowing for the association between the FCHL allele and the X2 allele were calculated assuming linkage equilibrium between alleles detected using the microsatellite tandem repeat and the *XmnI*/FCHL haplotype. In pedigree 3, FCHL segregated with the X1 allele with no recombinant events. The negative lod score for this family is a consequence of computation of likelihoods assuming linkage disequilibrium between FCHL and the X2 allele. This apparent anomaly may result from the marrying-in at a higher level in the pedigree of a normal individual carrying the X2 allele, as 1 in 5 normal individuals carries at least one X2. It may also be due to an ancestral recombinant event. The proband of pedigree 1 has two X2 alleles, one of which segregates with the disease and one which does not. This may have arisen by a similar mechanism to that seen in pedigree 3. All other pedigrees showed segregation of FCHL with the X2 allele.  $\theta$ , Recombination fraction.

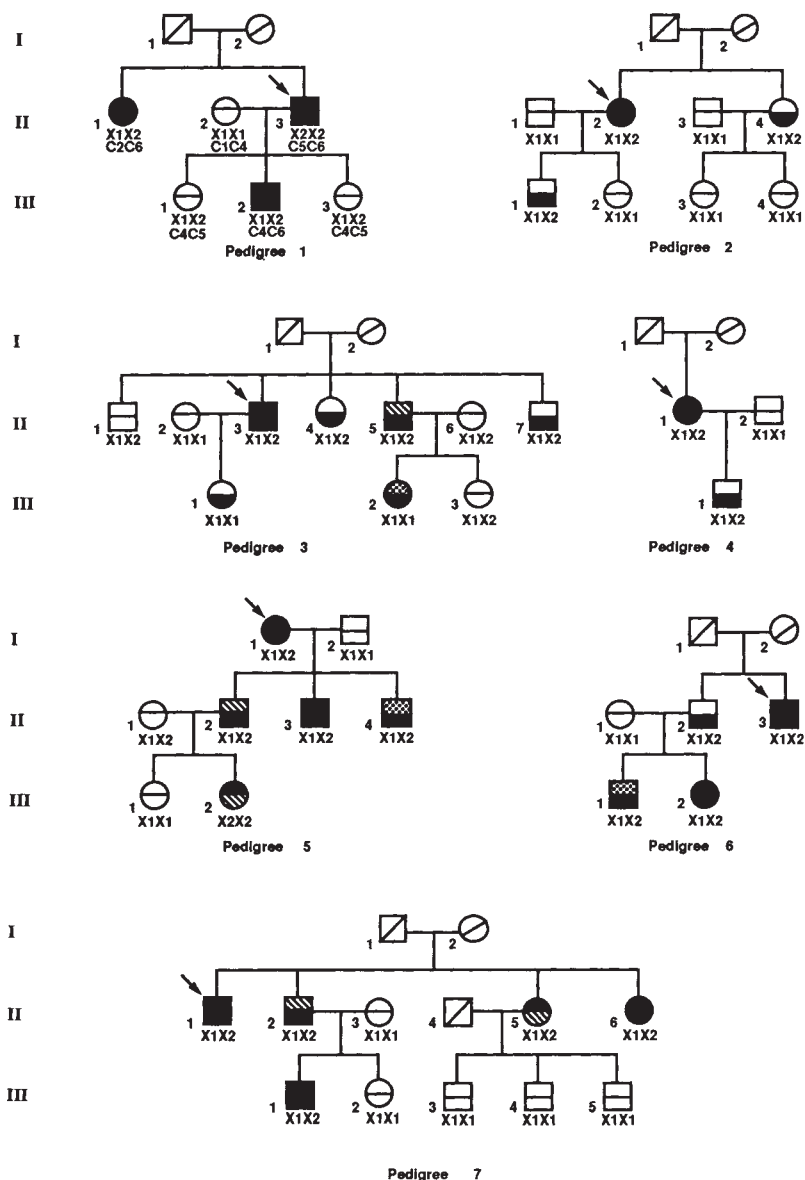
level or a triglyceride level (or both) above the ninety-fifth percentile. All individuals were sampled twice in the fasted state. Only if both samples were above the ninety-fifth percentile were they deemed to be affected. Individuals with secondary causes of hyperlipidaemia were excluded, as were those with familial tendon xanthomatosis. As FCHL is thought to be dominantly inherited, FCHL patients are assumed to be heterozygotes. Homozygotes are expected to be extremely rare as FCHL has a population prevalence of about 1%. Normolipidaemic controls had cholesterol and triglyceride levels below the seventy-fifth percentile. Eight of sixteen probands and seven of forty controls were found to carry the X2 polymorphism. Chi-squared analysis was carried out using the ASSOC program (version 2.2; gift of Jurg Ott<sup>11</sup>). Significant association was seen between the *XmnI* RFLP and the FCHL phenotype ( $\chi^2 = 6.29$ ,  $P = 0.012$ ) when sources of genotypic association other than linkage disequilibrium (for example, epistasis) were excluded (Table 1b). When our data and that of the original association study<sup>8</sup> are combined, the estimated frequencies of the X2 allele on normal chromosomes is 0.094 and on FCHL chromosomes is 0.391.

Individuals heterozygous for lipoprotein lipase (LPL) deficiency also show an FCHL phenotype. Indeed, a defect of the LPL gene may occur in up to a fifth of FCHL families<sup>12</sup>. As both the LPL gene and the apo-AI-CIII-AIV gene cluster

are implicated in the pathogenesis of FCHL, the disorder is genetically heterogeneous. We assembled the eight FCHL families in which the proband carried the 6.6-kb *XmnI* (X2) allele<sup>8</sup>. Seven of these pedigrees were used to test for linkage with the AI-CIII-AIV gene cluster (Fig. 1); because of hyperlipidaemia in the spouse of the proband, the other was excluded to avoid confounding the analysis by the introduction of other genes that may cause hyperlipidaemia. An individual from each pedigree, four probands (pedigrees 1, 2, 4 and 6) and three first degree relatives (pedigrees 3, 5 and 7) had small dense LDL which was assayed by density gradient ultracentrifugation and non-denaturing gradient gel electrophoresis<sup>13</sup>.

In assembling families for analysis, individuals with secondary hyperlipidaemia were excluded, as were children (aged < 16 years)<sup>2</sup>. Relatives of the proband were defined as 'affected' if either the cholesterol or the triglyceride level (or both) were above the ninety-fifth percentile. 'Unaffected' relatives were those in whom both of these levels were below the seventy-fifth percentile. Relatives with intermediate lipid levels were excluded before linkage analysis<sup>14</sup>. Children of family members whose spouse had intermediate or raised lipid levels were also excluded. Lipid results for all family members included in the linkage analysis are shown in Table 2. The 'affected' individuals show variability in their lipid levels, both within and between

FIG. 1. Family members with cholesterol and triglyceride levels <75th percentile are designated 'normal'; members with either a cholesterol or triglyceride level >95th percentile, or both >95th percentile are designated as 'affected'. Other members are designated 'intermediate' and are excluded from linkage analysis and not shown. Proband is marked with an arrow. X1 represents the 8.3-kb allele and X2 the 6.6-kb allele of the *XmnI* RFLP in the apo-AI-CIII-AIV gene cluster<sup>8</sup>. In pedigree 1, C1-6 represent alleles of a (CTTT)<sub>41-44</sub> microsatellite tandem repeat polymorphism at the 3' end of an *Alu* sequence in the third intron of the apo-CIII gene. This was detected by performing a polymerase chain reaction (PCR) with oligonucleotides 5'-GCAGTCCTAGGCCGCGCCG-3' and 5'-AGCCGAGATGGCACCCTGC-3', and including 1  $\mu$ Ci [<sup>32</sup>P]dATP (3,000 Ci mmol<sup>-1</sup>) in the reaction. PCR was performed by initially denaturing for 5 min at 94 °C, then using 30 cycles of 1 min at 94 °C, 2 min at 64 °C and 2 min at 72 °C. PCR products were analysed on 6% polyacrylamide sequencing gels and autoradiographed for 1-9 days. ■, Cholesterol > 95th percentile; □, triglyceride > 95th percentile; ▨, cholesterol and triglyceride > 95th percentile; ▩, cholesterol > 90th percentile; ▪, triglyceride > 90th percentile; ▫, cholesterol and triglyceride > 90th percentile; ▬, cholesterol > 75th percentile; ▭, triglyceride > 75th percentile; □, cholesterol and triglyceride < 75th percentile.



families. This phenotypic variability, which may be due to the influence of other genetic and environmental factors, is in keeping with the original description of FCHL<sup>2</sup>.

Six of the seven pedigrees were informative with the *Xmn*I RFLP. The remaining pedigree, where the proband (a parent) was homozygous for the X2 allele, was informative with a novel polymorphism of a (CTTT)<sub>41-44</sub> 'microsatellite' tandem repeat (Fig. 1) at the 3' end of an *Alu* sequence in the third intron of the apo-CIII gene<sup>15</sup>.

The log likelihood ratios (or lod scores) were calculated using the LINKAGE program and are shown in Table 3 (ref. 16). FCHL was linked to the apo-AI-CIII-AIV locus with no recombinant events; the peak lod score was  $\hat{z} = 6.86$  at  $\theta = 0.0$  ('lod -1.0' support interval<sup>17</sup>,  $\theta = 0.0-0.074$ ). The results confirm that these FCHL pedigrees have a defect in or close to the AI-CIII-AIV locus which results in hyperlipidaemia.

Although several major defects in this gene cluster have previously been reported, none results in an FCHL phenotype<sup>18</sup>. Apo-AI, the major structural apolipoprotein of high density lipoprotein (HDL), is unlikely to be implicated in the pathogenesis of FCHL as the main reported effect of mutations in this gene is a lowering of HDL levels<sup>19</sup>. The functions of apo-CIII and apo-AIV are less well understood. However, apo-AIV may facilitate the transfer of apo-CII to chylomicrons<sup>20</sup>; because apo-CII is an essential cofactor for the hydrolysis of triglyceride-rich particles by lipoprotein lipase, a defect in apo-AIV may cause hypertriglyceridaemia. Apo-CIII inhibits the action of lipoprotein lipase and hepatic lipase as well as the uptake of VLDL and chylomicron remnants by the liver, and thus might affect triglyceride and cholesterol levels<sup>21,22</sup>. Moreover, an increase in VLDL apo-CIII occurs in some hypertriglyceridaemic patients<sup>23</sup>.

Our results indicate that the AI-CIII-AIV gene cluster is the major locus for FCHL in this subset of families. In both this study and that of Hayden *et al.*<sup>4</sup>, the X2 allele was found in half of all FCHL families. A defect in this locus therefore accounts for a metabolic disorder which is an important predisposing cause of premature coronary heart disease in the general population. Identification of the causal mutation will allow early detection of those at risk and enable effective preventive measures to be undertaken. □

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## Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimaera

Susan R. Watson, Christopher Fennie & Laurence A. Lasky

Department of Immunobiology, Genentech, 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

NEUTROPHIL-mediated inflammation is involved in a number of human clinical manifestations, including the adult respiratory distress syndrome, multi-organ failure and reperfusion injury<sup>1</sup>. One way of inhibiting this type of inflammatory response would be to block competitively the adhesive interactions between neutrophils and the endothelium adjacent to the inflamed region<sup>2</sup>. The lectin-containing<sup>3,4</sup> murine adhesion molecule gp90<sup>MEL</sup>, the homing receptor, is found on all leukocytic cells, including neutrophils<sup>5</sup>. MEL 14, a monoclonal antibody directed against this adhesion molecule, blocks lymphocyte traffic to lymph nodes<sup>6</sup> and extravasation of neutrophils from blood to inflammatory sites<sup>7</sup>. Here we show that administration to mice of a soluble immunoglobulin chimaera containing the murine homing receptor extracellular domain significantly decreases the number of neutrophils that migrate to the peritoneum in response to the inflammatory irritant thioglycollate. These results indicate that soluble forms of a single type of adhesion molecule, the homing receptor, could be clinically effective compounds for the inhibition of neutrophil-mediated inflammation.

We have previously described the production of a soluble immunoglobulin (IgG) chimaera of the murine homing receptor

(HR) (mHRLEC-IgG; Fig. 1a)<sup>8</sup>. This molecule consists of the extracellular domain of the murine homing receptor ligated to the hinge, CH2 and CH3 domains of the human IgG-1 heavy chain (Fig. 1a). The advantages of using an IgG chimaera<sup>8,9</sup> include its ability to form an immunoglobulin-like dimer with a potentially higher avidity and serum half life, as well as improved purification through IgG-binding protein A-Sepharose chromatography. The mHRLEC-IgG chimaera was shown to block the binding of lymphocytes to peripheral lymph node endothelium *in vitro*, to stain this type of endothelium immunohistochemically<sup>8</sup>, and specifically to interact with a glycoprotein ligand of molecular weight 50,000 (50K) synthesized by peripheral and mesenteric lymph node high endothelial cells<sup>10</sup>. These results indicated that the mHRLEC-IgG chimaera specifically recognizes an endothelial ligand for leukocyte adhesion. To produce large quantities of this molecule for *in vivo* studies, we increased its production in permanently transfected mammalian cell lines (293 cells) by methotrexate amplification<sup>11</sup> and purified the material from cell-conditioned supernatants by protein A-Sepharose chromatography. It can be seen in Fig. 1b that the material in cell-conditioned medium (arrow) was purified to apparent homogeneity (Fig. 1c) at 5 mg l<sup>-1</sup> of medium. These cells therefore were an efficient and stable source of the mHRLEC-IgG chimaera.

The MEL 14 monoclonal antibody is an antibody that binds to the lectin domain of the murine homing receptor<sup>12</sup>; it efficiently blocks cell binding to peripheral and mesenteric lymph node endothelium *in vitro*<sup>13</sup> and inhibits transport to these lymph nodes *in vivo*<sup>6</sup>. Figure 2a shows the inhibition by this antibody of the passage of <sup>51</sup>Cr-labelled lymphocytes to peripheral and mesenteric lymph nodes, but not to Peyer's patches. In similar experiments to test the effect of the mHRLEC-IgG chimaera on lymphocyte traffic *in vivo* we found that the chimaera inhibited transport of lymphocytes to peripheral and mesenteric lymph nodes (Fig. 2a) presumably by binding



FIG. 1 Construction and purification of the mHREc-IgG chimaera. *a*, Protein domains of the homing receptor<sup>3,4</sup> are shown: S, signal sequence; L, lectin domain; EGF, epidermal growth factor-like domain; CB, complement binding-like domains; TMD, transmembrane domain. The mHREc-IgG consists of the extracellular domain of the murine homing receptor (where LEC represents the L, EGF-like and CB-like domains) ligated to the hinge (H), CH2 and CH3 domains of the human IgG1 (refs 8, 9). Details of the construction have been presented<sup>8</sup>. *b*, Coomassie blue-stained unpurified tissue culture supernatants run on 7.5% SDS-PAGE; *c*, Coomassie blue-stained material obtained after single-step purification on protein A-Sepharose and run on 7.5% SDS-PAGE. Molecular weight calibration is shown on the left (in thousands).

**METHODS.** Using the calcium phosphate procedure, 293 cells (adenovirus E1A-transformed human embryonic kidney cells) were transfected with 10  $\mu$ g mHREc-IgG plasmid<sup>8</sup>, 1  $\mu$ g G418-resistance plasmid and 1  $\mu$ g of a plasmid containing the complementary DNA encoding dihydrofolate reductase. Cells were selected in G418 and the incorporated plasmids co-amplified in the presence of 3  $\mu$ M methotrexate<sup>11</sup>. Permanent lines expressing high levels of the mHREc-IgG chimaera as assayed by enzyme-linked immunosorbent assay (ELISA)<sup>8</sup> were scaled up in roller bottles and serum free cell-conditioned supernatants collected. Amicon-concentrated supernatants were passed over protein A-Sepharose columns, bound material was eluted with 0.1 M acetic acid, 150 mM NaCl, immediately neutralized with 3M Tris-HCl, pH 9.0, and dialysed extensively against Dulbecco's phosphate-buffered saline. Final mHREc-IgG concentrations were determined by ELISA<sup>8</sup>.

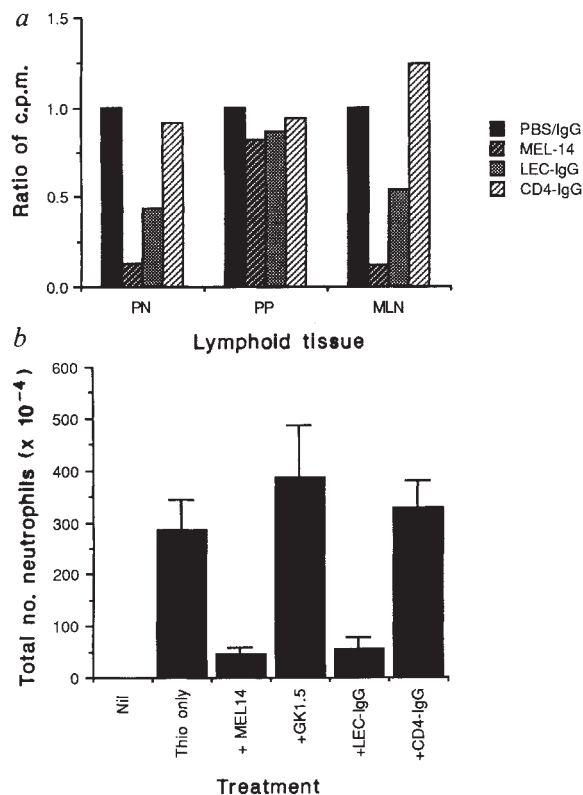
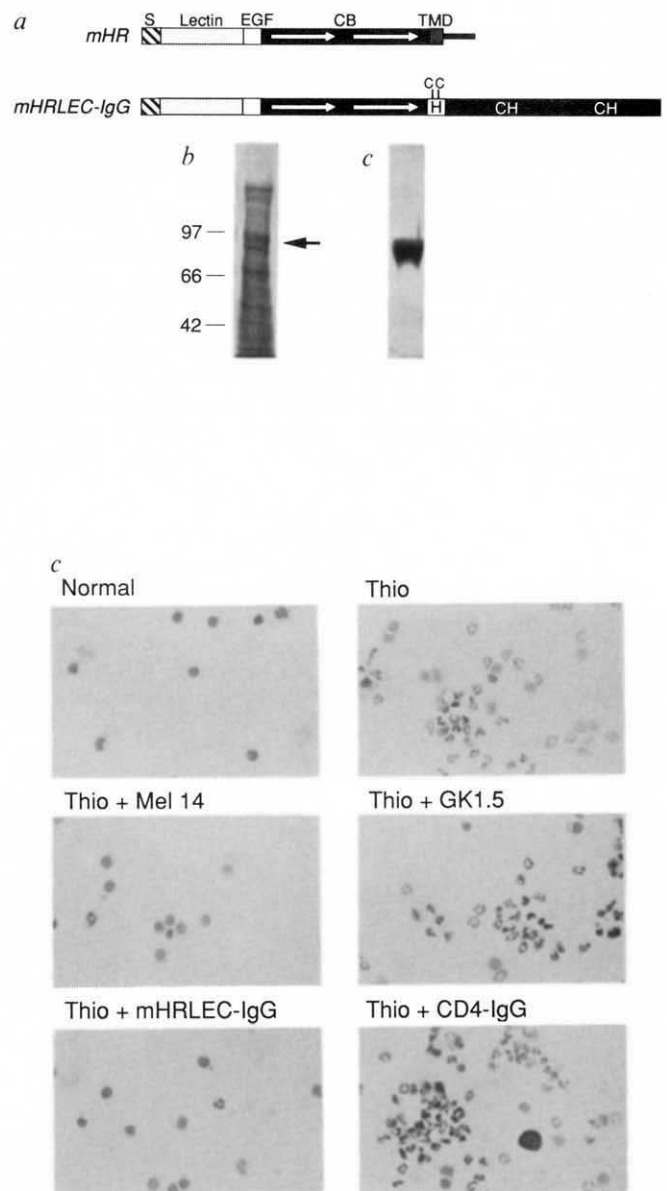


FIG. 2 Effects of MEL 14 and mHREc-IgG on *in vivo* leukocyte traffic. *a*, Inhibition of <sup>51</sup>Cr-labelled lymphocyte traffic to peripheral lymph nodes (PN), Peyer's patch lymph nodes (PP) and mesenteric lymph nodes (MLN). *b*, Inhibition of neutrophil extravasation into the peritoneum in response to thioglycollate by MEL 14 and mHREc-IgG. Abbreviations as in *c*. *c*, Cytospin preparations of peritoneal lavages from animals treated with thioglycollate (Thio) in the presence of MEL 14, mHREc-IgG, or control antibody (GK 1.5) or control IgG chimaera (CD4-IgG).

**METHODS.** Female BALB/c mice were cervically dislocated, their mesenteric lymphocytes removed aseptically and a single-cell suspension prepared. After washing, cells were labelled with sodium 51-chromate and spun through fetal calf serum, washed, spun through Ficoll, and washed twice. Cells (>80% viable by trypan-blue exclusion) were suspended to  $2 \times 10^7$  per ml. Equal volumes of cells were mixed with MEL 14, mHREc-IgG or CD4-IgG at 100  $\mu$ g protein per  $2 \times 10^6$  cells. Age and sex-matched BALB/c mice were intravenously injected with cells plus proteins or cells plus PBS in 200- $\mu$ l

volumes. Each animal received between 10,000 and 30,000 c.p.m. Two hours later, the animals were killed and blood, PN, PP, MLN, lung, liver, spleen, thymus and kidneys removed. Organs were counted in a gamma counter. Results in *a* are representative of 5 experiments in which each group contained at least 5 animals. They are expressed as the ratio of counts found in the PN, PP or MLN relative to untreated (PBS) controls. Control groups had 350–450 c.p.m. in PN, 700–800 in MLN and 900–1,300 in PP. For neutrophil traffic, female BALB/c mice were injected concomitantly with 1 ml thioglycollate intraperitoneally and 100  $\mu$ g each protein intravenously. Two hours later, animals were killed and blood samples removed. Peritoneal lavage was performed by injecting 10 ml Dulbecco's PBS containing 0.1% BSA and 10 units ml<sup>-1</sup> heparin, massaging the peritoneal wall, and removing the injected liquid. Total cells were counted using a Coulter counter, cytospin prepared, stained with Dif-Quik and differentially counted. All groups contained at least 4 animals, with the exception of the control group which only contained 2.

competitively to a ligand(s) for the homing receptor expressed on the endothelium of these organs<sup>8,10,14</sup>. As movement to Peyer's patch is not inhibited, lymphocyte migration must be organ-specific<sup>8,13,14</sup>. We conclude that the mHRLEC-IgG chimaera can effectively inhibit lymphocyte traffic through lymph node high endothelium *in vivo*.

We next tested whether the mHRLEC-IgG chimaera could inhibit neutrophil inflammation. Thioglycollate is an effective inducer of neutrophil inflammation<sup>7,15</sup>, and in a two-hour course of exposure of the mouse peritoneum to thioglycollate we found an increase of ~100-fold in the number of neutrophils by peritoneal lavage and microscopic examination of cytospin preparations. Intravenous administration of MEL 14 antibody simultaneously with thioglycollate exposure significantly decreases the number of neutrophils found in the peritoneum, whereas a control anti-L3T4 antibody (GK1.5) did not. This indicates that the MEL 14 adhesion system could be involved in acute neutrophil-mediated inflammation of the peritoneum<sup>7,15</sup>. Intravenous administration of the mHRLEC-IgG chimaera (100 µg per animal; corresponding to ~30 µg per ml of blood) at the same time as thioglycollate exposure significantly inhibited (~75%) neutrophil influx; a control IgG chimaera containing the human CD4 molecule<sup>9</sup> had no inhibitory effect. Counts of circulating neutrophils in animals receiving the mHRLEC-IgG chimaera showed that this effect was not merely due to a decrease in total neutrophil number. Our results are consistent with the hypothesis that neutrophil inflammation into the peritoneum makes use of the homing receptor adhesion system<sup>7,15</sup>, that the ligand(s) on endothelial cells that is recognized by neutrophils during this inflammatory event may be similar or identical to that found on the lymph node endothelium, and that this

inflammatory response may be blocked by a soluble dimeric form of the homing receptor.

The dose response and time course were next investigated to evaluate the clinical potential of the mHRLEC-IgG chimaera. Administration of as little as 10 µg of mHRLEC-IgG per animal (corresponding to ~3 µg per ml blood) resulted in a significant decrease of neutrophil influx into the peritoneum (Fig. 3a). Doses of 10 µg per animal MEL 14 antibody gave no significant effects on peritoneal influx of neutrophils, possibly because the levels of leukocyte cell-surface homing receptor antigen were higher than those of endothelial cell homing receptor ligand(s). We also analysed the relative kinetics of peritoneal accumulation of neutrophils in the presence of either the MEL 14 antibody or the mHRLEC-IgG chimaera. As can be seen from Fig. 3b, both reagents inhibited the accumulation of neutrophils effectively at the 2-hour time point, but the inhibition of neutrophil influx at 4 hours seemed much weaker. This could be explained by the disappearance of the chimaera, but half-life studies (S.R.W., unpublished results) show that this molecule is relatively long-lived (half life about 50 hr) in the mouse circulation. This result suggests that homing receptor-mediated adhesion contributes to the initial influx of neutrophils during inflammation but that other mediators of adhesion are activated later that dominate the inflammatory pathway.

The blocking of neutrophil influx by the IgG chimaera is consistent with the possibility that the homing receptor ligand on the endothelium adjacent to the peritoneal irritant is similar or identical to that in the peripheral and mesenteric lymph node high endothelium. So it is possible that the ligand, presumably predominantly carbohydrate in nature<sup>10</sup>, is induced during inflammation. At least one of the lymph node ligands for this adhesion molecule is a glycoprotein of molecular weight ~50,000 whose interaction with the homing receptor requires sialic acid<sup>10</sup>. The induction event could therefore involve changes in protein synthesis, protein glycosylation, or both. The fact that neutrophils are the predominant cell type found in the inflammatory lesion and that other leukocytic cells have comparable levels of homing receptor on their surfaces, indicates that inflammatory responses might be mediated by a combination of both adhesive and cell-signalling events.

The use of soluble cell-surface glycoproteins as inhibitors of viral infection<sup>9,16</sup> or immune function<sup>17</sup> has been proposed as a basis for development of new therapeutic drugs. Our data demonstrate that a soluble form of a specific cell adhesion molecule, the murine homing receptor, is an effective inhibitor of acute inflammatory responses *in vivo*, although the ability of this molecule to alter the normal traffic patterns of cells may be an important clinical side-effect. This work significantly extends earlier results showing that monoclonal antibodies directed against adhesion molecules were anti-inflammatory<sup>2,18-20</sup>. It will be interesting to test other soluble adhesion molecules such as the integrins, their ligands<sup>16</sup> and other lectin cell adhesion molecules (for example, endothelial leukocyte adhesion molecule (ELAM)<sup>21</sup> and gmp140/platelet activation-dependent granule external membrane protein)<sup>22,23</sup> for their capacity to inhibit acute or chronic inflammatory responses. Combinations of soluble adhesion molecules may ultimately be required in view of the fact that the decreased inhibition due to our mHRLEC-IgG chimaera at 4 h after thioglycollate injection is consistent with the induction kinetics for ELAM expression<sup>21</sup>. It is possible that this lectin cell adhesion molecule becomes the dominant adhesion molecule directing the later stages of neutrophil influx. Such studies should coordinate the rational design of anti-inflammatory reagents based on competitive blocking of leukocyte-endothelial cell-adhesive interactions. □

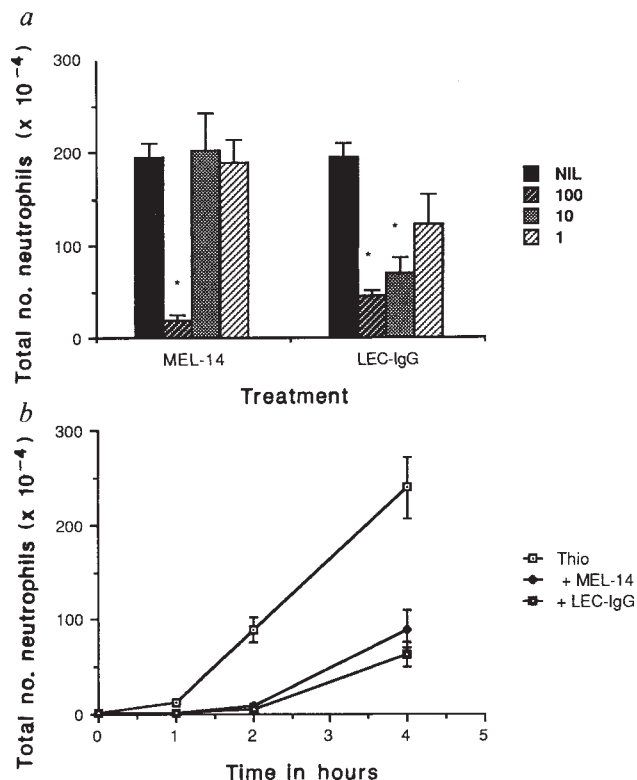


FIG. 3 Effect of mHRLEC-IgG dose and time on peritoneal neutrophil influx. METHODS. a, As in Fig. 2b, except that mice received 100, 10 or 1 µg of either the MEL 14 monoclonal antibody or purified mHRLEC-IgG. Asterisks indicate data significant at  $P > 0.01$  or more. Error bars show s.e.m.; all groups had 6 animals. b, As in Fig. 2b, except that animals receiving a PBS control, or 100 µg MEL 14 antibodies, or 100 µg of the mHRLEC-IgG chimaera were killed at 1, 2 and 4 h after thioglycollate injection and examined for intraperitoneal neutrophil inflammation.

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## Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene

Tatsuo Shioda, Jay A. Levy & Cecilia Cheng-Mayer

Cancer Research Institute, Department of Medicine,  
University of California, San Francisco, California 94143-0128, USA

**STRAINS of human immunodeficiency virus type 1 (HIV-1) display a high degree of biological heterogeneity which may be linked to certain clinical manifestation of AIDS. They vary in their ability to infect different cell types<sup>1-3</sup>, to replicate rapidly and to high titre in culture<sup>4-6</sup>, to down-modulate the CD4 receptor<sup>7,8</sup>, and to cause cytopathic changes in infected cells<sup>7,9-11</sup>. Some of these *in vitro* properties correlate with pathogenicity of the virus *in vivo*<sup>11,12</sup>. To map the viral determinants of the cellular host range of HIV-1, recombinant viruses were generated between biologically active molecular clones of HIV-1 isolates showing differences in infection of primary peripheral blood macrophages and established T-cell lines. We report here that a specific region of the envelope gp120 gene representing 159 amino-acid residues of glycoprotein gp120 seems to determine macrophage tropism, whereas an overlapping region representing 321 amino-acid residues determines T cell-line tropism. These studies provide a basis for relating functional domains of the HIV-1 *env* gene to pathogenic potential.**

HIV-1<sub>SF2</sub>, an isolate recovered from peripheral blood mononuclear cells (PMC) of an infected individual with candidiasis<sup>13</sup>, replicates well in established T-cell lines, such as Hut78 and Jurkat, but cannot productively infect primary peripheral blood macrophages<sup>11,14</sup>. HIV-1<sub>SF162mc</sub>, an isolate from the brain of a patient with toxoplasmosis, replicates efficiently in primary peripheral blood macrophages, but cannot infect established T-cell lines<sup>8,14</sup>. The genomes of HIV-1<sub>SF2</sub> and HIV-1<sub>SF162</sub> have been molecularly cloned and sequenced<sup>14-16</sup>. Biological properties of viruses obtained from the molecular clones (HIV-1<sub>SF2mc</sub>, HIV-1<sub>SF162mc</sub>) are the same as those of the parental viruses<sup>14</sup>. Both viruses replicate well and to comparable titres in PMC, but differ in their infectivity for primary macrophages and established T-cell lines (Fig. 1).

In a previous study<sup>14</sup> we generated recombinant viruses between HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub> by exchanging DNA fragments of their proviral genomes. These studies indicated that a 3.2 kilobase (kb) *EcoRI-XhoI* fragment, containing the sequence encoding the C-terminal 35 amino-acid residues of

*env*, the *tat*, *rev*, *upu* and *env* gene products and the N-terminal 36 or 38 amino-acid residues of the *nef* gene product, determines HIV-1 macrophage and T cell-line tropisms<sup>14</sup> (Fig. 2a, R5 and R6). To identify the specific gene or genomic region that controls this differential tropism, additional recombinant viruses have been generated by exchanging smaller sequences within this 3.2-kb region by methods described previously<sup>14,17</sup>.

The genomic structures and cellular host range properties of the recombinant viruses first generated are summarized in Fig. 2a. All the recombinant viruses grew to comparable titres in PMC. R7, R9, R11 and R13 grew well in primary peripheral blood macrophages, but not in Hut78 cells, showing the same cellular host range as that of the parental HIV-1<sub>SF162mc</sub> (R2) and of R5. R7 and R9 have the 1.8-kb *EcoRI-SspI* sequence of HIV-1<sub>SF162mc</sub>; R11 has the 2.9-kb *StuI-EcoRI* sequence of HIV-1<sub>SF162mc</sub> (Fig. 2a). Therefore, the overlapping 0.71-kb *StuI-SspI* sequence, corresponding to the central 236 amino-acid residues of gp120, seems to control the macrophage tropism of HIV-1 (Fig. 2a).

We consistently observed, however, that R11 grew to lower titres than the parental HIV-1<sub>SF162mc</sub> (R2) in the macrophage cultures, whereas R7, R9, and R13 showed no substantial differences in titre or kinetics of viral replication compared with R2 (Fig. 2a). Therefore a 0.25-kb *DraIII-StuI* sequence of HIV-1<sub>SF162mc</sub>, corresponding to 78 amino-acid residues of gp120, seems to be required for efficient infection of primary macrophages.

R8, R10, R12 and R14 have the same cellular host range as that of parental HIV-1<sub>SF2mc</sub> (R1) and R6 (Fig. 2a). These recombinant viruses productively infected the Hut78 T-cell line, but not peripheral blood macrophages. The common 0.96-kb *DraIII-SspI* sequence shared by R8, R10, R12 and R14, and corresponding to the central 321 amino-acid residues of gp120 of HIV-1<sub>SF2mc</sub>, most probably controls the tropism for this established T-cell line.

In these studies, R8, R10 and R12 showed delayed growth kinetics in Hut78 cells (Fig. 2a), whereas R14, the parental HIV-1<sub>SF2mc</sub> (R1), and R6 grew well in the same cell line. This phenotype of R8, R10 and R12 is stable as viruses recovered from Hut78 cultures infected with R8, R10 or R12 again showed the delayed growth kinetics in Hut78 cells (data not shown). Therefore, efficient infection of this T-cell line seems to require a large 2.3-kb *EcoRI-BsmI* HIV-1<sub>SF2mc</sub> sequence containing the first coding exon of the *tat* and *rev* genes, *upu*, the entire gp120 gene sequence, and the sequence encoding the N-terminal 101 amino-acid residues of gp41.

To confirm the data obtained above, and to identify the minimal genetic sequences required to confer T-cell or macrophage tropism on HIV-1, five additional recombinant

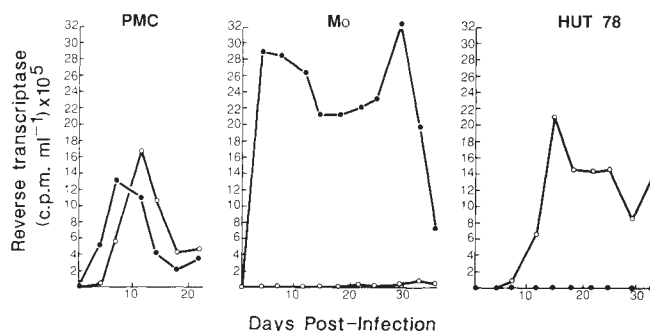
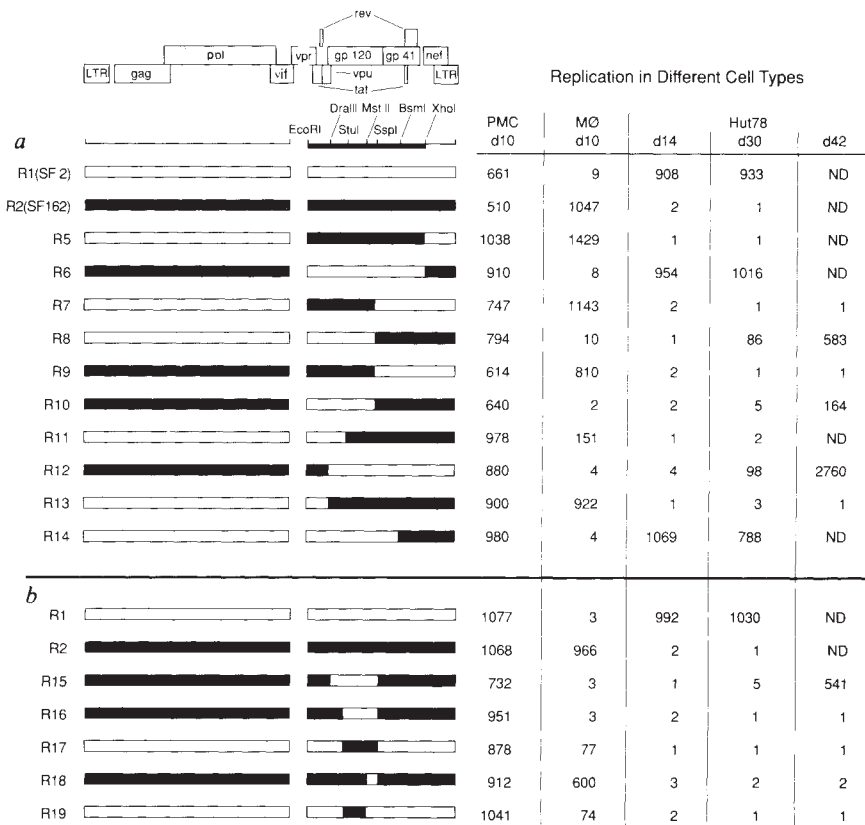


FIG. 1 Replication of viruses generated from molecular clones of HIV-1<sub>SF2</sub> (HIV-1<sub>SF2mc</sub>) and HIV-1<sub>SF162</sub> (HIV-1<sub>SF162mc</sub>) in peripheral blood mononuclear cells (PMC), primary peripheral blood macrophages (Mφ), and the established human T-cell line Hut78. PMC, Mφ, or Hut78 cells were infected with 10<sup>6</sup> c.p.m. of reverse transcriptase activity of HIV-1<sub>SF2mc</sub> or HIV-1<sub>SF162mc</sub>. Culture supernatants of these infected cells were assayed for reverse transcriptase activity at 3-4 day intervals, as described<sup>14,25</sup>. ○, HIV-1<sub>SF2mc</sub>; ●, HIV-1<sub>SF162mc</sub>.



**FIG. 2** The genomic structures and biological properties of HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub> recombinant viruses. Open bars indicate DNA sequences from HIV-1<sub>SF2mc</sub> and closed bars those from HIV-1<sub>SF162mc</sub>. Using a common internal *EcoRI* site at the midpoint of both viral genomes and *EcoRI* sites in the 5' and 3' flanking cellular sequences, two principal fragments were obtained by *EcoRI* digestion of the biologically active phage  $\lambda$  clones of HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub> (ref. 14). The 5' *EcoRI* fragments contain cellular DNA sequences and the 5' long terminal repeat (LTR), *gag*, *pol* and *vif* genes of either HIV-1<sub>SF2mc</sub> or HIV-1<sub>SF162mc</sub>. The 3' *EcoRI* fragments are composed of the *tat*, *rev*, *vpu*, *env*, *nef* genes and the 3' LTR. Our studies concentrated on the 3' *EcoRI* fragments. Restriction enzyme cleavage sites used for generating recombinant 3' DNA are indicated (*DraIII*, *StuI*, *MstII*, *SspI*, *BsmI* and *XhoI*). Recombinant viruses were recovered by cotransfection of the 5' and 3' fragments into human RD-4 cells followed by co-cultivation with PMC from seronegative individuals<sup>14,17</sup>, and were inoculated at  $10^6$  c.p.m. of reverse transcriptase activity into PMC, M $\phi$  and Hut78. For replication in PMC and M $\phi$ , reverse transcriptase activity ( $\times 10^3$  c.p.m. ml<sup>-1</sup> of culture fluid) at 10 days post-infection are presented. For replication in Hut78 cells, reverse transcriptase activity ( $\times 10^3$  c.p.m. ml<sup>-1</sup> of culture fluid) at 14 days, 30 days and 42 days post-infection are presented. All data are representative of at least three independent experiments. ND, not determined. **a**, Structures and properties of R1–R14; **b**, structures and properties of R15–R19. R15, R16, and R18 contain the 0.96-kb *DraIII*–*SspI*, 0.71-kb *StuI*–*SspI*, and 0.24-kb *MstII*–*SspI* fragments of HIV-1<sub>SF2mc</sub> gp120, respectively, in the HIV-1<sub>SF162mc</sub> genomic background. R17 and R19



have the 0.71-kb *StuI*–*SspI* and 0.48-kb *StuI*–*MstII* fragments of HIV-1<sub>SF162mc</sub> gp120, respectively, in the HIV-1<sub>SF2mc</sub> genomic background.

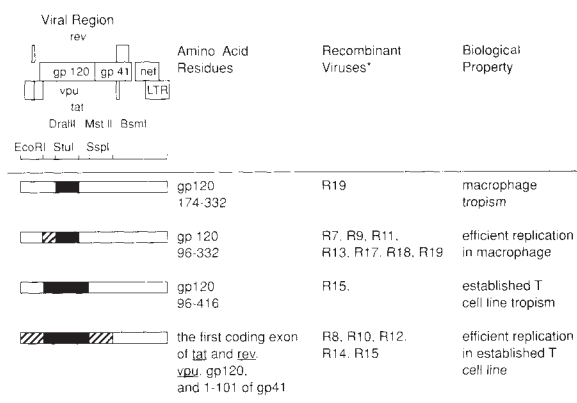
viruses were generated and analysed (Fig. 2b, R15–R19). All recombinant viruses grew in PMC to titres comparable with those of parental HIV-1<sub>SF2mc</sub> (R1) and HIV-1<sub>SF162mc</sub> (R2). R17 replicated in macrophage cultures but not in the Hut78 T-cell line, confirming that the main determinant for macrophage tropism lies within the 0.71-kb *StuI*–*SspI* fragment of HIV-1<sub>SF162mc</sub>. The finding that R19 also replicated to the same extent as R17 in macrophage cultures but not in Hut78 cells indicates that the smaller 0.48-kb *StuI*–*MstII* fragment, representing 159 amino-acid residues of gp120 of HIV-1<sub>SF162</sub>, contains the minimal genetic sequences necessary to confer macrophage tropism on HIV-1.

Both R17 and R19 consistently grew to titres similar to those of R11 (Fig. 2a), and thus lower than the parental HIV-1<sub>SF162mc</sub>

in the macrophage cultures. This supports our conclusion that the 0.25-kb *DraIII*–*StuI* sequence of HIV-1<sub>SF162mc</sub> is necessary for efficient replication in primary macrophages. As no substantial differences in titre or kinetics of viral replication were observed between R17 and R19, and between HIV-1<sub>SF162mc</sub> (R2) and R18, we concluded that the 0.24-kb *MstII*–*SspI* fragment, representing 77 amino-acid residues of gp120 of HIV-1<sub>SF162mc</sub>, does not have any role in determining macrophage tropism of HIV-1 (Fig. 2b).

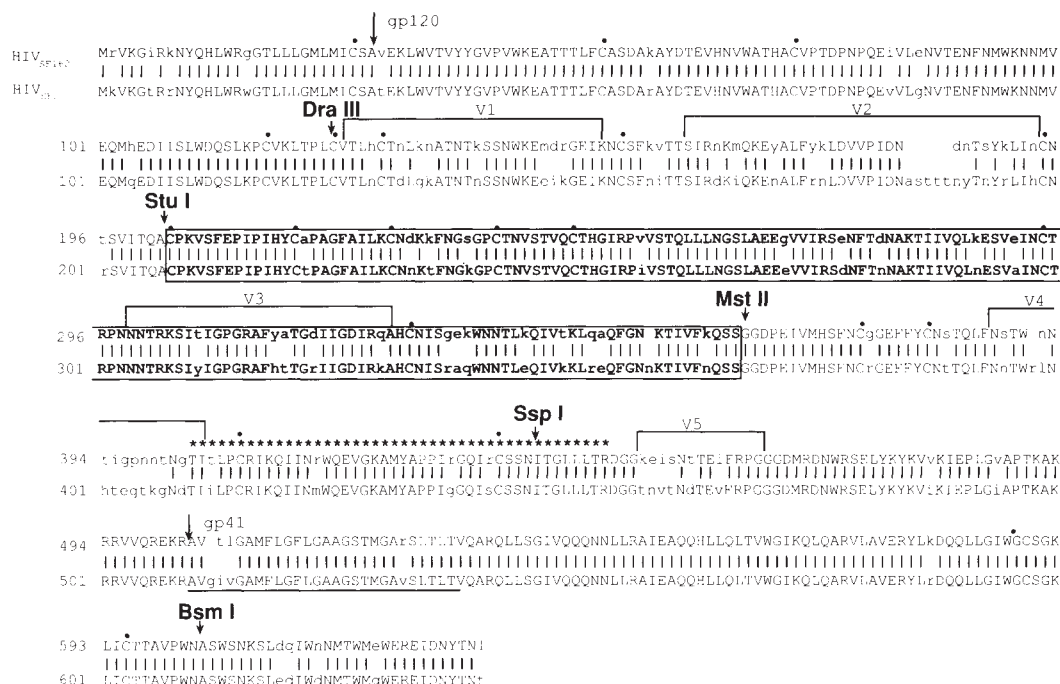
The biological properties of R15 and R16 confirmed our finding that the principal determinants for infection of established T-cell lines are within the 0.96-kb *DraIII*–*SspI* sequence of gp120 of HIV-1<sub>SF2mc</sub> (Fig. 2b). Furthermore, the delayed growth of R15 in Hut78 cells supports our conclusion that regions other than the 0.96-kb *DraIII*–*SspI* fragment are necessary for efficient replication in T-cell lines. Figure 3 summarizes the regions and amino-acid residues of gp120 found to be required for infection and efficient replication in primary macrophages and established T-cell lines.

A comparison of the predicted amino-acid sequences between HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub> gp120 and the N-terminal 131 amino-acid residues of gp41 is shown in Fig. 4. Differences were clustered primarily within the five hypervariable regions of gp120 (ref. 18) and in the putative gp41 fusion domain<sup>19</sup>. The envelope region that seemed to contain the principal determinant of the macrophage tropism of HIV-1 corresponds to the *StuI*–*MstII* fragment encompassing the V3 hypervariable region of gp120. Efficient replication in primary macrophages requires a 0.25 kb *DraIII*–*StuI* fragment containing sequences encoding the V1 and V2 regions of gp120. The envelope region that correlated with infection of established T-cell lines corresponds to the *DraIII*–*SspI* fragment that contains the sequences encoding the V1, V2, V3 and V4 hypervariable regions and the CD4 binding domain. No known regulatory sequences, such as *tat*, *rev* or the *rev* responsive element<sup>20,21</sup>, are located within the regions identified.



**FIG. 3** Viral genetic regions responsible for specific biological properties of HIV-1. Viral regions essential for macrophage tropism or established T-cell line tropism are indicated by closed bars. Viral regions necessary for efficient replication in macrophage culture or the established T-cell line Hut78 are indicated by hatched bars. \* Recombinant viruses that provided the data.

FIG. 4 A comparison of the predicted amino-acid sequences of HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub> gp120 and the N-terminal 131 amino-acid residues of gp41. The five hypervariable regions (V1, V2, V3, V4 and V5) are bracketed. The CD4-binding domain<sup>24</sup> is marked with asterisks, and the gp41 fusion peptide domain is underlined. Dots show cysteine residues. Potential cleavage sites<sup>26</sup> are indicated by long thin arrows. Restriction enzyme cleavage sites used to generate recombinant viruses are indicated by small arrows (*Dra*III, *Stu*I, *Mst*II, *Ssp*I and *Bsm*I). The amino-acid sequences corresponding to the 0.48-kb *Stu*I-*Mst*II fragment, which is essential for macrophage tropism of HIV-1<sub>SF162mc</sub>, are in bold letters and boxed.



Recombinant viruses have been used in previous studies to map determinants of host range tropism<sup>22,23</sup>. In these studies, however, only one of the molecular clones used in the generation of recombinant virus was biologically active. Our use of recombinant viruses generated between two biologically active molecular clones of HIV-1 that show differences in T-cell and macrophage tropism, provided the opportunity to examine more definitely the effects of reciprocal exchange of envelope regions associated with specific cell tropism on infection of different cell types.

We were able to map the viral determinant of macrophage tropism to a relatively small region of gp120 that does not include the CD4 binding domain<sup>24</sup>. A relatively larger overlapping portion of envelope gp120, however, is required for efficient infection of Hut78 cells (Fig. 3). This may reflect the involvement of multiple discontinuous envelope determinants in infection of this cell type. Furthermore, we could not exclude the possibility that certain element(s) in the *vpu* gene and the first coding exon of the *tat* and *rev* genes have some role in determining the rate of replication. The *vpu* gene in HIV-1<sub>SF2mc</sub> is, however, truncated<sup>17</sup> and is therefore unlikely to have a functional role. The roles of the *tat* and *rev* genes of HIV-1<sub>SF2mc</sub> in its kinetics and level of replication in T-cell lines need to be examined.

Our finding that specific regions in gp120 alone are sufficient to determine certain tropisms suggests that the entry process is responsible for the differences in host range tropism observed between HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub>. This agrees with our observation that transfection of the biologically active molecular clone of HIV-1<sub>SF162</sub> into Hut78 cells leads to the production of infectious virions (data not shown). Some of the 67 amino-acid differences between HIV-1<sub>SF2mc</sub> and HIV-1<sub>SF162mc</sub> in the *Dra*III-*Ssp*I fragment of gp120 therefore most probably affect the receptor binding and/or post-binding penetration process in a cell type-specific manner. These results provide valuable new information on viral gene-function relationships that could be involved in HIV pathogenesis, including that in the brain. The findings could also be helpful for developing new therapeutic strategies. □

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## Phosphorylation of non-muscle caldesmon by p34<sup>cdc2</sup> kinase during mitosis

Shigeko Yamashiro\*, Yoshihiko Yamakita\*, Hiroshi Hosoya\*† & Fumio Matsumura\*

\* Department of Molecular Biology and Biochemistry, Nelson Hall, Busch Campus, Rutgers University, Piscataway, New Jersey 08855-1059, USA and † The Tokyo Metropolitan Institute for Medical Science, Tokyo, Japan

ONE of the profound changes in cellular morphology which occurs during mitosis is a massive alteration in the organization of the microfilament cytoskeleton<sup>1,2</sup>. This change, together with other mitotic events including nuclear membrane breakdown, chromosome condensation and formation of mitotic spindles, is induced by a molecular complex called maturation promoting factor<sup>3-5</sup>. This consists of at least two subunits, a polypeptide of relative molecular mass 45,000-62,000 (*M*, 45-62K) known as cyclin, and

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a 34K catalytic subunit which has serine/threonine kinase activity and is known as cdc2 kinase<sup>6-9</sup>. Non-muscle caldesmon, an 83K actin- and calmodulin-binding protein, is dissociated from microfilaments during mitosis, apparently as a consequence of mitosis-specific phosphorylation<sup>10</sup>. We now report that cdc2 kinase phosphorylates caldesmon *in vitro* principally at the same sites as those phosphorylated *in vivo* during mitosis, and that phosphorylation reduces the binding affinity of caldesmon for both actin and calmodulin. Because caldesmon inhibits actomyosin ATPase, our results suggest that cdc2 kinase directly causes microfilament reorganization during mitosis.

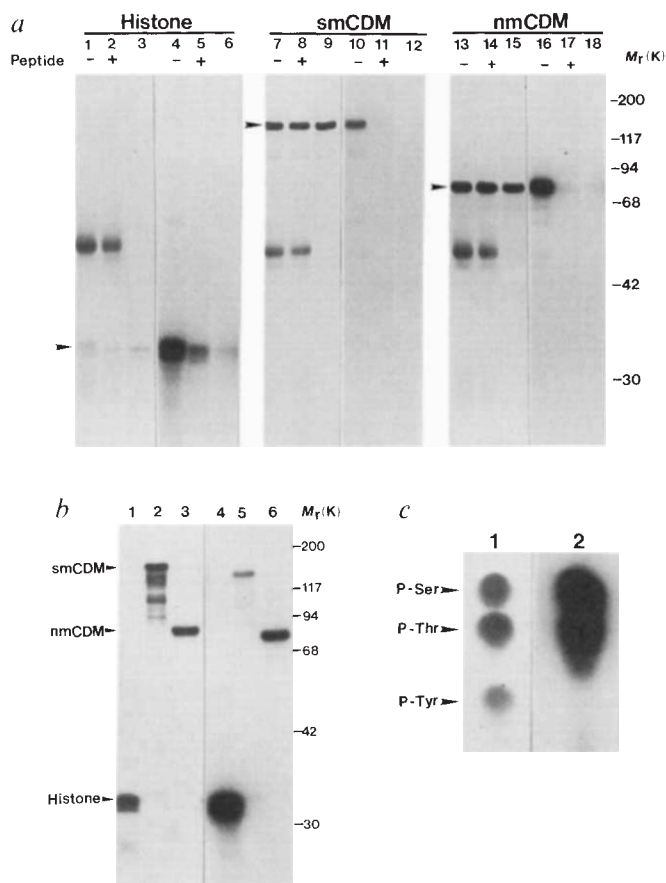


FIG. 1 Phosphorylation of caldesmon by cdc2 kinase, and phosphoamino-acid analysis. *a*, Phosphorylation by human cdc2 kinase examined by Coomassie blue staining (lanes 1-3, 7-9 and 13-15) and by corresponding autoradiography (lanes 4-6, 10-12 and 16-18). smCDM, Smooth-muscle caldesmon; nmCDM, non-muscle caldesmon. The addition of competitive antigenic C-terminal peptide during immunoprecipitation of human cdc2 kinase abolished the phosphorylation (lanes 5, 11, 17). Autophosphorylation controls were shown in lanes 3, 6 (histone H1); 9, 12 (smCDM); and 15, 18 (nmCDM). *b*, Phosphorylation of histone H1 (lanes 1 and 4), smCDM (lanes 2 and 5) and nmCDM (lanes 3 and 6) by purified *Xenopus* MPF. Lanes 1-3, Coomassie-blue staining; lanes 4-6, autoradiography. *c*, Phosphorylation of caldesmon at serine and threonine residues *in vivo* during mitosis. Lane 1, marker phosphoamino acids detected by ninhydrin; lane 2, autoradiography. METHODS. *a, b*, Kinase reactions were performed at 30 °C for 20 min in 50 mM Tris-HCl, pH 7.5 containing 10 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 1 mM DTT, 500 nM peptide inhibitor to A-kinase (SIGMA), 100 μM ATP (0.5 μCi [<sup>32</sup>P] ATP) and either 0.5 μg histone H1 (Boehringer Mannheim) or 2 μg caldesmon in a total volume of 20 μl. Human cdc2 kinase was prepared by immunoprecipitation either in the absence or presence of C-terminal peptides according to ref. 17, and 5 μl human cdc2 kinase or 1 μl *Xenopus* kinase was added to each reaction. Reactions were terminated at 20 min by the addition of 6 μl 5×SDS sample buffer and analysed by SDS-PAGE. *c*, Phosphoamino-acid analysis was performed as described<sup>18</sup>. Caldesmon labelled *in vivo* with <sup>32</sup>P was prepared by immunoprecipitation as described<sup>10</sup>, and further purified by SDS-PAGE.

Both human and *Xenopus* cdc2 kinase preparations were tested for caldesmon kinase activity. Human cdc2 kinase, prepared by immunoprecipitation from HeLa cell mitotic extracts using anti-cdc2 antibody (raised against the C terminus of human cdc2 (ref. 11)), phosphorylates both smooth-muscle and non-muscle caldesmons, as well as histone H1 (Fig. 1*a*, lanes 4, 10, 16). The phosphorylation of these proteins was blocked when human cdc2 kinase was prepared in the presence of competing antigenic peptide (lanes 5, 11, 17). Phosphorylation *in vitro* of caldesmon also occurs with highly purified *Xenopus* MPF (ref. 12) (Fig. 1*b*). Because these two preparations are totally independent, it is unlikely that caldesmon was phosphorylated by impurities contaminating these cdc2 preparations. It is noteworthy that the antibody, unlike that raised against PSTAIR, does not precipitate 'cdc2-like' kinases<sup>5,13</sup>.

Other evidence for the phosphorylation *in vitro* of caldesmon by cdc2 is the co-purification of the histone H1 kinase activity of cdc2 with the caldesmon kinase activity. During the isolation of caldesmon kinase activity from mitotic HeLa cell extracts by sequential column chromatography (Sephacryl S-300, poly-L-lysine-agarose, phenyl-Sepharose and reactive yellow Y-86 in this order), the fractions containing the histone H1 kinase activity show caldesmon kinase activity (data not shown). Mitotic extracts also have an additional caldesmon kinase activity different from that of cdc2. Poly-L-lysine-agarose

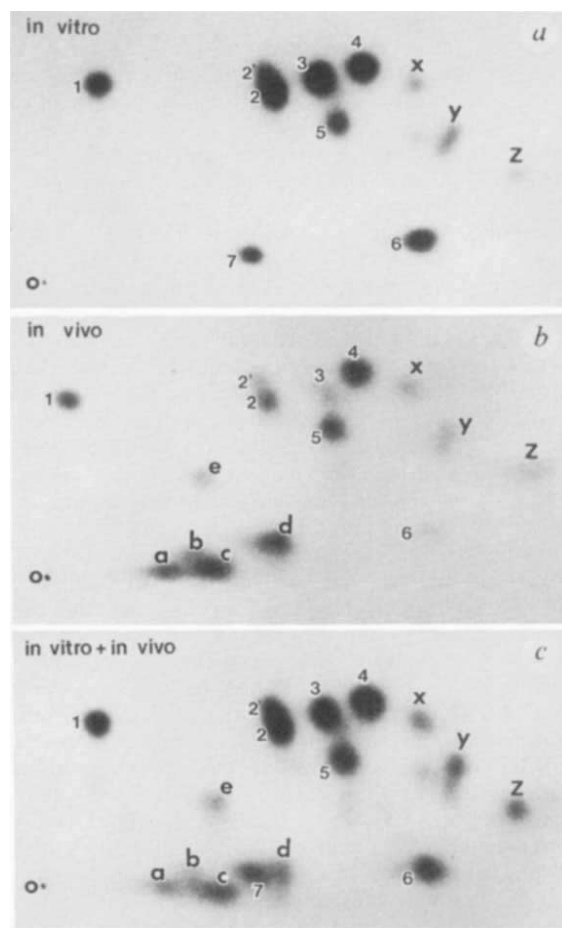


FIG. 2 Two-dimensional phosphopeptide map of caldesmon. *a*, Phosphorylated *in vitro* by cdc2 kinase; *b*, phosphorylated *in vivo*; *c*, a mixture of forms phosphorylated *in vivo* or *in vitro*.

METHODS. Caldesmon labelled *in vitro* by human cdc2 kinase was prepared as described in Fig. 1 and further purified by SDS-PAGE. Caldesmon labelled *in vivo* was prepared by immunoprecipitation as described<sup>10</sup>. Tryptic phosphopeptides were prepared with TPCK-trypsin (Worthington) and mapped in two dimensions as described<sup>19</sup> with modification<sup>20</sup>.





(1.5 kb) contains a termination codon and encodes a 297-amino-acid C-terminal peptide. Sequence comparison has revealed that A16 and D3 show extensive similarity with N- and C-terminal regions of chick caldesmon, respectively (Fig. 3).

Among the six (chick) or seven (rat) (Ser/Thr)-Pro sequences found in these caldesmons, five occur in the same positions of the sequence when the two sequences are aligned for maximum homology. All five sites are located in the 25K (based on SDS gels) C-terminal actin- and calmodulin-binding domain of chick smooth-muscle caldesmon<sup>14,15</sup>, which is generated by chemical cleavage at Cys 580 with 2-nitro-5-thiocyanatobenzoic acid (NTCB). The same cleavage of rat non-muscle caldesmon should produce a C-terminal fragment only slightly smaller than 25K.

To examine whether mitosis-specific phosphorylation by cdc2 indeed occurs at the C terminus, both chick smooth-muscle and rat non-muscle caldesmon were phosphorylated *in vitro* by human cdc2 kinase and then these caldesmons, along with rat caldesmon phosphorylated *in vivo*, were chemically cleaved at cysteine residues by NTCB treatment. As reported previously<sup>15</sup>, chick caldesmon contains two cysteine residues and thus five fragments were generated (Fig. 4a, lane 3). Autoradiographic data indicate that the C-terminal 25K fragment is phosphorylated (Fig. 4a, lane 4) although the N-terminal and central regions are not.

As expected, NTCB cleavage of rat non-muscle caldesmon produced two fragments of 60K and 20K, which are assigned as the N and C termini, respectively. The 20K fragment of rat non-muscle caldesmon exhibits actin- and calmodulin-binding ability (data not shown), as does the 25K C-terminal fragment of chick caldesmon, confirming the assignment of this fragment to the C terminus. Autoradiography has revealed that the C-terminal 20K fragment of nonmuscle caldesmon contains the sites predominantly phosphorylated both *in vitro* by cdc2 kinase (Fig. 4a, lane 8) and *in vivo* (Fig. 4a, lane 10).

Phosphorylation of caldesmon by cdc2 kinase reduces its affinity for both actin and calmodulin. An actin binding assay (Fig. 4b) revealed that  $0.30 \pm 0.05 \mu\text{mol}$  cdc2-phosphorylated caldesmon ( $n = 3$ ) precipitates with  $12 \mu\text{mol}$  of actin (lane 2), whereas  $0.65 \pm 0.05 \mu\text{mol}$  control unphosphorylated caldesmon ( $n = 3$ ) is pelleted (lane 4). A calmodulin binding assay using a calmodulin-Sepharose column (Fig. 4c) has shown that after phosphorylation with cdc2 a large amount of caldesmon was eluted from the column during washing, whereas most of the unphosphorylated caldesmon remained bound to the column and was eluted with the EGTA-containing solution.

Caldesmon is a regulatory protein implicated in the control of the motility and contractility of non-muscle and smooth-muscle cells<sup>16</sup>, and inhibits actomyosin ATPase when it binds to actin. The dissociation of caldesmon from actin caused by phosphorylation by cdc2 is thus likely to release the inhibition of actomyosin ATPase activity. This may in turn induce a large change in actomyosin organization during mitosis. Our findings therefore strongly suggest that cdc2 kinase directly controls the changes in microfilament assembly seen during mitosis. □

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## Altered tyrosine 527 phosphorylation and mitotic activation of p60<sup>c-src</sup>

Shubha Bagrodia, Isaac Chackalaparampil, Thomas E. Kmiecik\* & David Shalloway†

Section of Biochemistry, Molecular and Cell Biology and Department of Pathology, Cornell University, Ithaca, New York 14853, USA

THE tyrosine kinase activity of p60<sup>c-src</sup>, the protein product of the *c-src* gene, increases during mitosis<sup>1,2</sup>; this may be important in initiating at least some of the cellular changes that occur during this phase of the cell cycle. Although there is evidence that p60<sup>c-src</sup> is phosphorylated at several sites during mitosis, phosphorylation *in vitro* does not increase its kinase activity<sup>2,3</sup>. We now report that the kinase activity of a p60<sup>c-src</sup> mutant with residue tyrosine 527 changed to phenylalanine does not change during the cell cycle, suggesting that changes in the phosphorylation state of this residue may be responsible for the activation of p60<sup>c-src</sup> at mitosis. Although changes in phosphorylation at Tyr 527 cannot be detected with the wild-type protein we find that phosphorylation at Tyr 527 of a mutant with reduced kinase activity decreases threefold during mitosis. On the basis of these results we suggest that activation of p60<sup>c-src</sup> at mitosis results from decreased phosphorylation on Tyr 527, and that p60<sup>c-src</sup> may be or may activate the kinase that phosphorylates Tyr 527.

The activity of p60<sup>c-src</sup> from unsynchronized cells is decreased 10–20-fold by Tyr 527 phosphorylation<sup>4–6</sup> and increased two-to-threefold by Tyr 416 phosphorylation<sup>7</sup>. We began evaluating the significance of these phosphorylations for mitosis-specific activation of p60<sup>c-src</sup> by analysing tryptic phosphopeptide maps of wild-type and mutant (Tyr 416 and/or Tyr 527 changed to Phe) p60<sup>c-src</sup> from unsynchronized and nocodazole-arrested (mitotic) NIH 3T3-derived *src*-overexpressor cells (Fig. 1). Apart from the predicted changes in tyrosine phosphorylation<sup>4</sup>, the phosphorylation patterns were identical. The mutations did not interfere with the three p34<sup>cdc2</sup>-mediated mitosis-specific phosphorylations at Thr 34, Thr 46 and Ser 42, and about half of both the wild-type (Fig. 3a) and mutant (data not shown) immunoprecipitated *c-src* proteins had retarded electrophoretic mobilities during mitosis, presumably caused by hyperphosphorylation.

Tyr 527 was still phosphorylated during mitosis (Fig. 1e; see ref. 1). However, this analysis does not accurately measure phosphorylation levels because phosphopeptides are neither generated nor recovered quantitatively. Small changes in phosphorylation of Tyr 527, sufficient to account for the mitotic increase in p60<sup>c-src</sup> kinase activity, could have been undetected.

The effects of mutation on mitotic activation were measured in the immune complex kinase assay (Fig. 2; Table 1). The rate at which wild-type p60<sup>c-src</sup> phosphorylated enolase increased about fourfold during mitosis (see Ref. 1). The mutant (Tyr 527 changed to Phe) showed no mitotic increase. This could simply indicate that the activity of the mutant had already been maximally stimulated. However, mitotic activation was also

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\* Present address: Frederick Cancer Research and Development Center, Advanced Bioscience Laboratories, Frederick, Maryland 21702, USA.

† To whom correspondence should be addressed.



blocked in the double mutant (Tyr 416 and Tyr 527 changed to Phe), which has sub-maximal kinase activity and thus could, in principle, be activated during mitosis. As mutation of Tyr 416 alone did not block mitotic activation (Fig. 2) and Tyr 416 was not phosphorylated in wild-type p60<sup>c-src</sup> from either unsynchronized or mitotic cells (Fig. 1a, e), we conclude that changes in Tyr 527 phosphorylation are required for mitotic activation of c-src kinase.

Mutation of Lys 295 to Arg, which renders p60<sup>c-src</sup> kinase-defective, does not measurably affect the phosphorylation of p60<sup>c-src</sup> from unsynchronized cells<sup>8,9</sup>. Surprisingly, this mutation reduced Tyr 527 phosphorylation in p60<sup>c-src(R295)</sup> from mitotic cells. As Tyr 527 was the only *in vivo* phosphotyrosine in both p60<sup>c-src</sup> and p60<sup>c-src(R295)</sup> (Fig. 1, and data not shown), its phosphorylation level was measured with anti-phosphotyrosine antibodies. Both p60<sup>c-src</sup> and p60<sup>c-src(R295)</sup> were immunoprecipitated from unsynchronized and mitotic cells and duplicate immunoblots were probed with either anti-Src or anti-phosphotyrosine antibodies to determine relative Tyr 527 phosphorylation levels (Fig. 3a). No significant change in the level of tyrosine phosphorylation of p60<sup>c-src</sup> during mitosis was detected, but a threefold decrease was measured with p60<sup>c-src(R295)</sup> (Table 1).

This decrease was confirmed by proteolytic analysis with *Staphylococcus aureus* V-8 protease (Fig. 3b). The ratio of phosphorylation within the amino-proximal V1, V3 and V4 fragments (containing phosphorylation sites Ser 12, Ser 17, Thr 34, Thr 46 and Ser 72) to phosphorylation within a carboxyl-proximal V2 fragment of mass 26,000 (containing Tyr 527) was 1.3–1.5 in both p60<sup>c-src</sup> and p60<sup>c-src(R295)</sup> from unsynchronized cells (reflecting extensive phosphorylation at Ser 17 and Tyr 527 and partial phosphorylation at Ser 12). This ratio was two- to threefold higher in mitotic retarded-mobility p60<sup>c-src</sup>, reflecting increased phosphorylation at the (amino-region) mitotic sites. In contrast, the ratio was 6-fold higher in mitotic retarded-mobility p60<sup>c-src(R295)</sup> owing to decreased Tyr 527 phosphorylation in addition to increased amino-region phosphorylation. The mitosis-specific decrease in tyrosine phosphorylation in p60<sup>c-src(R295)</sup> was also revealed by phosphoamino acid analysis (data not shown).

The mitosis-specific decrease in Tyr 527 phosphorylation in kinase-defective src may be caused by either decreased Tyr 527 kinase activity or increased phosphotyrosine phosphatase activity and might be similar to the mitosis-specific decrease in Tyr 15 phosphorylation in p34<sup>cdc2</sup> (refs 10, 11). The effect of phosphatase activity would be enhanced if the competing

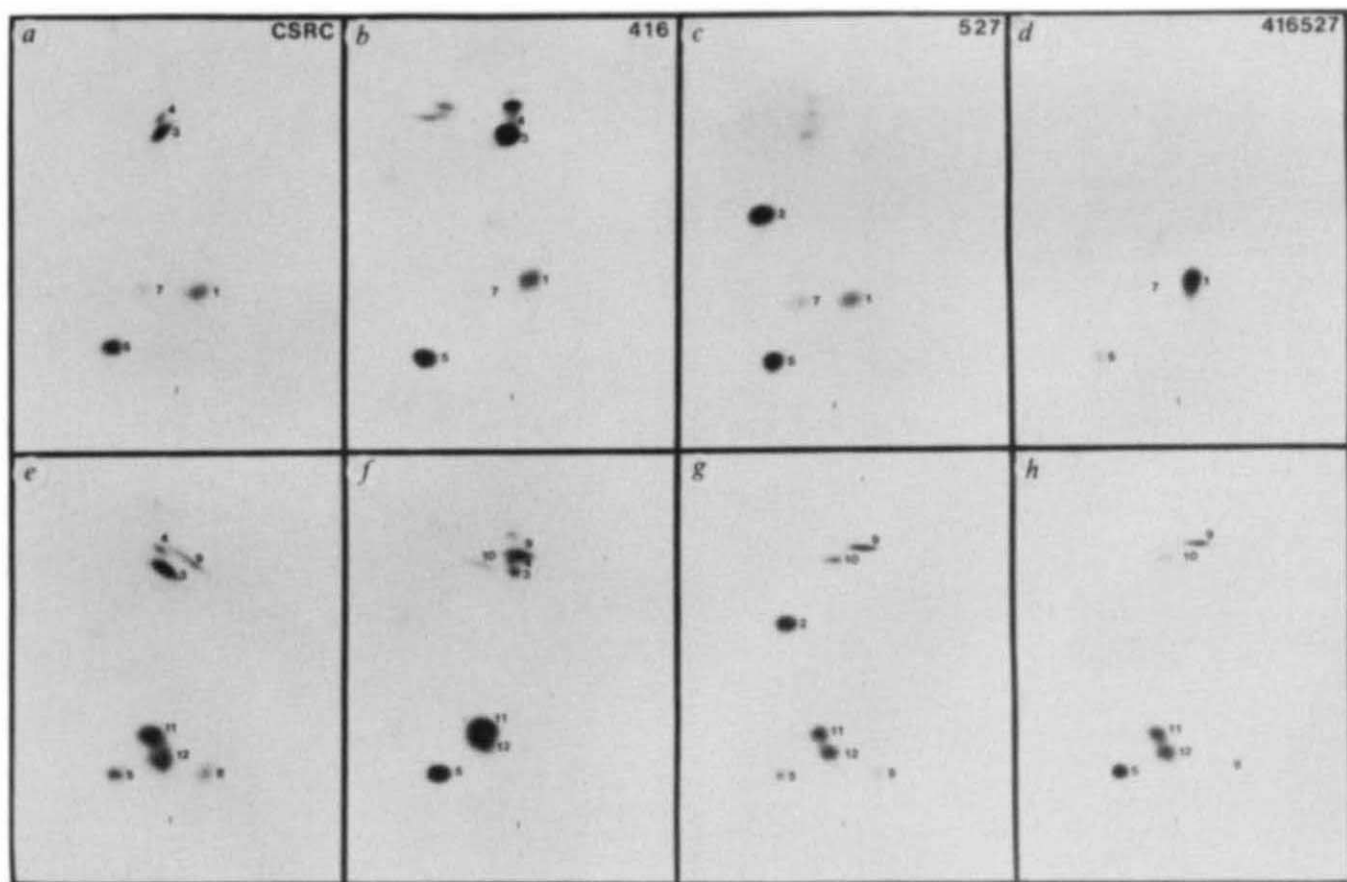


FIG. 1 Tryptic phosphopeptide maps of mutant c-src proteins from unsynchronized and mitotic cells. Wild-type and mutant p60<sup>c-src</sup> from [<sup>32</sup>P]-metabolically-labelled unsynchronized and (nocodazole-arrested) mitotic plasmid-transfected NIH 3T3 src-overexpresser cells were immunoprecipitated with monoclonal antibody 327 (ref. 14), purified by SDS-PAGE, digested with trypsin and analysed by two-dimensional electrophoresis and chromatography as described<sup>2</sup>. Less than 5 per cent of the immunoprecipitated proteins was endogenous p60<sup>c-src</sup>. a, e, Wild-type (wt) chicken p60<sup>c-src</sup>; b, f, mutant with Tyr 416 changed to Phe; c, g, mutant with Tyr 527 changed to Phe; d, h, double-mutant with Tyr 416 and Tyr 527 changed to Phe. a–d, Bands from unsynchronized cells; e–h, retarded-mobility mitotic upper bands. Spot designations<sup>2</sup>: 1, peptide containing phosphoserine 17; 2, phosphotyrosine 416; 3 and 4, phosphotyrosine 527; 5 and 6, phosphoserine 12; 7, phosphoserine 48; 8 and 11, phosphothreonine 46; 9, phosphoserine

72; 10, unidentified phosphoserine; and 12, phosphoserine 17 and phosphothreonine 34.

METHODS. Cell lines NIH(pMsrc/foc)B, NIH(pcsrc416/pSV2neo/MC)A, NIH(pcsrc527/foc/ep)B1 and NIH(pc416527/foc)A overexpressing wt and mutant chicken c-src proteins have been described<sup>4,15</sup>. All procedures were as described<sup>2</sup> with the following modifications. Cells were grown for 16 h (rather than 7.5 h) in 80% phosphate-free minimal essential medium (Flow Laboratories), 10% DMEM (Mediatech), 10% calf serum before lysis. Lysates were prepared in modified RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.02 M NaH<sub>2</sub>PO<sub>4</sub>, 1 mM PMSF, 2 mM EDTA, 50 mM NaF, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 30 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 14 mM 2-mercaptoethanol, 1 mM ZnCl<sub>2</sub> and 1 mM Na<sub>2</sub>MoO<sub>4</sub>). Tryptic digestions were performed in the presence of 100 μM TPCK (Sigma). Samples were spotted at arrowheads.



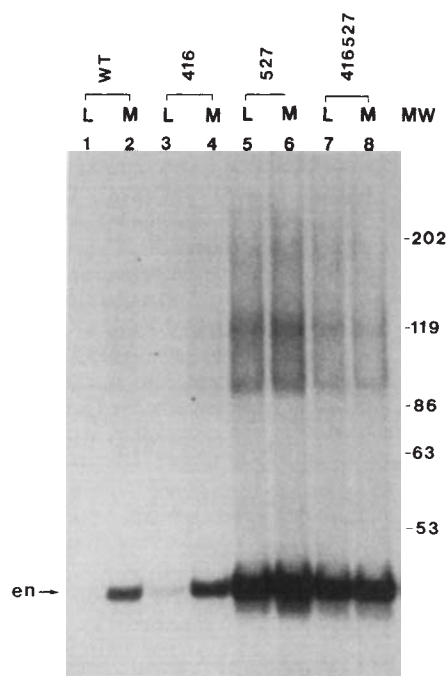
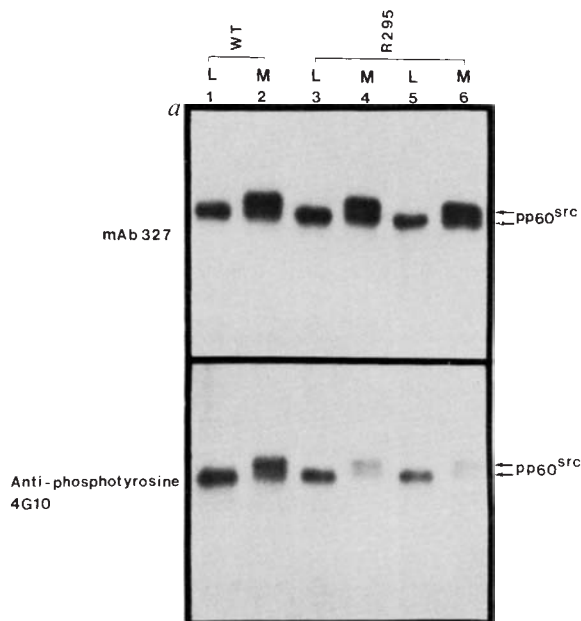


FIG. 2 Kinase activities of mutant *c-src* proteins from unsynchronized and mitotic cells. Lysates containing approximately equal amounts of unlabelled wt and mutant  $p60^{c-src}$  from unsynchronized (L) and mitotic (M) NIH 3T3-derived *src*-overexpresser cells were immunoprecipitated with monoclonal antibody 327. Immunoprecipitates were divided into portions; one (shown) was incubated with acid-denatured enolase and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and subjected to SDS-PAGE. The other (not shown) was immunoblotted and probed with antibody 327 to measure  $p60^{c-src}$  levels (levels were equal to within 30%). Relative specific kinase activities (Table 1) were calculated by dividing the amounts of  $[\text{P}]$ enolase (en) by the amounts of  $p60^{c-src}$ . Lanes: 1 and 2, wt chicken  $p60^{c-src}$ ; 3 and 4, mutant; with Tyr 416 changed to Phe; 5 and 6, mutant with Tyr 527 changed to Phe; 7 and 8, double-mutant with Tyr 416 and Tyr 527 changed to Phe. MW: Relative molecular mass markers (MW, in thousands) are shown to the right.

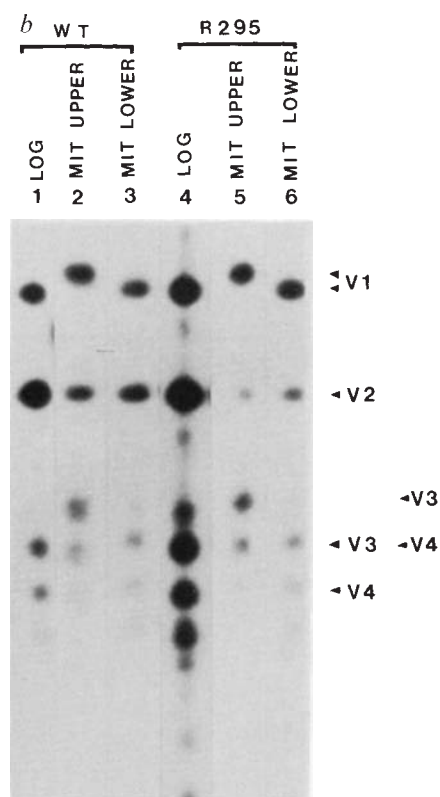
**METHODS.** Immunoprecipitations were as described in Fig. 1, except that cells were grown in normal medium without  $^{32}\text{P}$ . Kinase assays were performed as described<sup>1</sup> except that only one thirtieth as much  $p60^{c-src}$  was used, one wash with high-salt buffer was performed and incubations were for 4 min in 40  $\mu\text{l}$  of 20 mM HEPES (pH 7.0), 10 mM  $\text{MnCl}_2$ , 2 mM 2-mercaptoethanol, 1  $\mu\text{M}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (400 Ci  $\text{mmol}^{-1}$ ) and 4  $\mu\text{g}$  acid-denatured rabbit-muscle enolase. For immunoblotting after SDS-PAGE, proteins were electrotransferred to Immobilon-P (Millipore) (40 V, 12 h) in transfer buffer without methanol, probed with primary antibodies and washed as described<sup>16</sup>, except that filters were blocked for 16 h, 23  $^\circ\text{C}$  in 0.9% (w/v) NaCl, 20 mM Tris-HCl (pH 7.4), 5% bovine serum albumin, 1% ovalbumin. Filters were hybridized with 5  $\mu\text{g ml}^{-1}$  rabbit antimouse IgG (ICN), washed and incubated (1 h, 23 $^\circ\text{C}$ ) with  $^{125}\text{I}$ -labelled protein A (20 ng  $\text{ml}^{-1}$ ; 50  $\mu\text{Ci } \mu\text{g}^{-1}$ ; 10 ml TBS (ref. 16), ICN), washed and autoradiographed.

FIG. 3 Relative phosphorylation levels of wild-type and kinase-defective  $p60^{c-src}$  from unsynchronized and mitotic cells.

**a.** Relative levels of tyrosine phosphorylation. Unlabelled *src* proteins were immunoprecipitated with monoclonal antibody (mAb) 327 from unsynchronized (L) and mitotic (M) wt and mutant (with Lys 295 changed to Arg) *src*-overexpresser cells; equal portions were immunoblotted and probed with mAb 327 or anti-phosphotyrosine monoclonal antibody 4G10 (UBI) as for Fig. 2. Lanes: 1 and 2, wt  $p60^{c-src}$  (20 $\times$  endogenous  $p60^{c-src}$  expression); 3 and 4,  $p60^{c-src}(\text{R295})$  from low-level (10 $\times$  endogenous) expresser line; 5 and 6,  $p60^{c-src}(\text{R295})$  from high-level (60 $\times$  endogenous) expresser line. **b.** *S. aureus* V-8 protease analysis.  $[\text{P}]$ -metabolically labelled *src* proteins were immunoprecipitated with monoclonal antibody EC10 from unsynchronized and mitotic wt and mutant (Lys 295 changed to Arg) *src* overexpresser cells;  $p60^{c-src}$  SDS-PAGE bands were partially digested with 150 ng V8 protease as described<sup>1</sup>. Lanes: 1–3, wt  $p60^{c-src}$ ; 4–6, mutant; 1 and 4, normal-mobility bands from unsynchronized cells; 2 and 5, retarded-mobility (upper) bands from mitotic cells; 3 and 6, normal-mobility (lower) bands from mitotic cells. V1, 34K amino region V-8 fragment; V2, 26K carboxyl region fragment; V3, 18K amino region subfragment; V4, 16K amino region subfragment. NIH 3T3-derived overexpresser cell lines NIH(pMcsrc/foc)B and NIH(pCR295/pSV2neo/MC)C (10 $\times$ ) have been described<sup>2,13</sup>. NIH(pCR295/pSV2neo/cos)J (60 $\times$ ) was similarly co-selected. Western blots were performed as in Fig. 2. Exposure times (–70  $^\circ\text{C}$  with intensifying screen): **a**, 15 min; **b**, 1.5 d. Relative band intensities are compared in Table 1.



**b.** *S. aureus* V-8 protease analysis.  $[\text{P}]$ -metabolically labelled *src* proteins were immunoprecipitated with monoclonal antibody EC10 from unsynchronized and mitotic wt and mutant (Lys 295 changed to Arg) *src* overexpresser cells;  $p60^{c-src}$  SDS-PAGE bands were partially digested with 150 ng V8 protease as described<sup>1</sup>. Lanes: 1–3, wt  $p60^{c-src}$ ; 4–6, mutant; 1 and 4, normal-mobility bands from unsynchronized cells; 2 and 5, retarded-mobility (upper) bands from mitotic cells; 3 and 6, normal-mobility (lower) bands from mitotic cells. V1, 34K amino region V-8 fragment; V2, 26K carboxyl region fragment; V3, 18K amino region subfragment; V4, 16K amino region subfragment. NIH 3T3-derived overexpresser cell lines NIH(pMcsrc/foc)B and NIH(pCR295/pSV2neo/MC)C (10 $\times$ ) have been described<sup>2,13</sup>. NIH(pCR295/pSV2neo/cos)J (60 $\times$ ) was similarly co-selected. Western blots were performed as in Fig. 2. Exposure times (–70  $^\circ\text{C}$  with intensifying screen): **a**, 15 min; **b**, 1.5 d. Relative band intensities are compared in Table 1.



Tyr 527 kinase activity were diminished. Thus, if  $p60^{c-src}$  contributes to Tyr 527 phosphorylation, the observed reduction in its phosphorylation in the kinase-defective mutant (relative to wild type) is readily explained. This hypothesis is consistent with the finding that  $p60^{c-src}$  can intermolecularly phosphorylate Tyr 527 to some extent when expressed in yeast<sup>12</sup>. Alternatively, the *src* kinase may activate a distinct Tyr 527 kinase<sup>13</sup>.

In either case it is likely that both the kinase-defective and

wild-type *src* proteins are subject to similar mitosis-specific changes in Tyr 527 kinase and/or phosphatase activity (although the possibility that mutation of Lys 295 directly affects these activities cannot be excluded). Thus it is plausible that wild-type  $p60^{c-src}$  also undergoes some mitosis-specific decrease in Tyr 527 phosphorylation. As mutation of Tyr 527 to Phe increases  $p60^{c-src}$  kinase activity 20-fold (Table 1), a 20% decrease (undetectable within experimental error) in Tyr 527 phosphorylation could

TABLE 1 Relative kinase activities and phosphorylation levels

| Relative specific kinase activities* |                                  |         |                                        |         |
|--------------------------------------|----------------------------------|---------|----------------------------------------|---------|
|                                      | Unsynchronized                   |         | Mitotic                                |         |
| Wild-type                            |                                  | 1.0     |                                        | 4.4     |
| Tyr 527 → Phe                        |                                  | 20.0    |                                        | 20.0    |
| Tyr 416 → Phe/Tyr 527 → Phe          |                                  | 11.0    |                                        | 9.3     |
| Tyr 416 → Phe                        |                                  | 1.7     |                                        | 4.6     |
| Relative phosphorylation levels      |                                  |         |                                        |         |
|                                      | Relative phosphotyrosine levels† |         | Amino:carboxyl region phosphorylation‡ |         |
|                                      | Unsynchronized                   | Mitotic | Unsynchronized                         | Mitotic |
| Wild-type                            |                                  |         |                                        |         |
| Upper                                |                                  | 1.2     |                                        | 3.1     |
| Lower                                | 1.0                              | 1.2     | 1.3                                    | 1.5     |
| Lys 295 → Arg                        |                                  |         |                                        |         |
| Upper                                |                                  | 0.3     |                                        | 6.0     |
| Lower                                | 0.9                              | 0.3     | 1.5                                    | 3.6     |

\* Specific kinase activities of wt and mutant *src* proteins from unsynchronized and mitotic cells were calculated from the ratios of  $^{32}\text{P}$  incorporated into enolase relative to the amounts of immunoblotted  $\text{p60}^{\text{c-src}}$  in experiments of the type shown in Fig. 2. Values are geometric averages from four experiments and are normalized to the kinase activity of wt  $\text{p60}^{\text{c-src}}$  from unsynchronized cells. The difference between the wt and the mutant with Tyr 416 changed to Phe in unsynchronized cells is within the error range (standard fractional errors range from 5–30%).

† The relative stoichiometry of tyrosine phosphorylation in wt and mutant (Lys 295 changed to Arg) *src* proteins from unsynchronized and mitotic cells was determined from scintillation counting and densitometry of double-immunoblotting experiments like that shown in Fig. 3a. Values are geometric averages from 4 experiments (standard fractional errors are 60% for wild-type and 7% for mutant protein measurements).

‡ The ratio of total phosphorylations within the amino-proximal (mass 34,000) and carboxyl-proximal (26,000) regions of wt and mutant *src* proteins from unsynchronized and mitotic cells was determined from  $^{32}\text{P}$ -labelled V-8 digests like that shown in Fig. 3b. Upper, values for retarded mobility bands from mitotic cells; lower, values from normal-mobility bands present in unsynchronized and mitotic cells. Values are geometric averages from 3 experiments (standard fractional errors range from 12–26%).

account for the 4-fold increase in activity during mitosis.

It remains to be shown whether the  $\text{p34}^{\text{cdc}2}$ -mediated phosphorylations activate  $\text{p60}^{\text{c-src}}$  during mitosis and, if so, by what mechanism  $\text{p34}^{\text{cdc}2}$  may act either by decreasing the Tyr 527 phosphorylation rate or by increasing the Tyr 527 dephosphorylation rate. It is not known whether any of the  $\text{p34}^{\text{cdc}2}$ -mediated phosphorylations are critical for these effects. Immunoblotting and V-8 experiments show that phosphorylation at Tyr 527 of  $\text{p60}^{\text{c-src}}$  is suppressed during mitosis in both the normal and retarded-mobility forms (Table 1, and Fig. 3a, b; compare upper and lower bands). The normal mobility form contains phosphothreonine 46 and phosphoserine 72 but not phosphothreonine 34 (data not shown), indicating that at least Thr 34 phosphorylation is not required for decreased Tyr 527 phosphorylation. We are now exploring the roles of phosphorylation at Thr 46 and Ser 72. Should all these phosphorylations be irrelevant for activation, the possibility that a Tyr 527 kinase or tyrosine phosphatase is a target of  $\text{p34}^{\text{cdc}2}$  must be considered. □

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## Base sequence discrimination by zinc-finger DNA-binding domains

Jeannette Nardelli\*, Toby J. Gibson†, Christine Vesque\* & Patrick Charnay\*

\* Laboratoire de Génétique Moléculaire, CNRS D 1302, École Normale Supérieure, 46, rue d'Ulm, F-75230 Paris Cedex 05, France  
† European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 10-2209, D-6900 Heidelberg, Germany

**ZINC** fingers<sup>1,2</sup> constitute important eukaryotic DNA-binding domains, being present in many transcription factors<sup>3–5</sup>. The Cys<sub>2</sub>/His<sub>2</sub> zinc-finger class has conserved motifs of 28–30 amino acids which are usually present as tandem repeats<sup>1,2</sup>. The structure of a Cys<sub>2</sub>/His<sub>2</sub> zinc finger has been determined by nuclear magnetic resonance<sup>6</sup>, but details of its interaction with DNA were not established. Here we identify amino acids governing DNA-binding specificity using *in vitro* directed mutagenesis guided by similarities between the zinc fingers of transcription factors Sp1 (ref. 7) and Krox-20 (ref. 8). Krox-20 is a serum-inducible transcription activator<sup>8,9</sup> which is possibly involved in the regulation of hindbrain development<sup>10</sup>; it contains three zinc fingers similar to those of Sp1 (refs 7, 8, 11) and binds to a 9-base-pair target sequence which is related to that of Sp1 (ref. 9). Our results show that each finger spans three nucleotides and indicate two positions in Krox-20 zinc fingers that are important for base-pair selectivity. Modelling with molecular graphics suggests that these residues could bind directly with the bases and that other amino acid–base contacts are also possible.

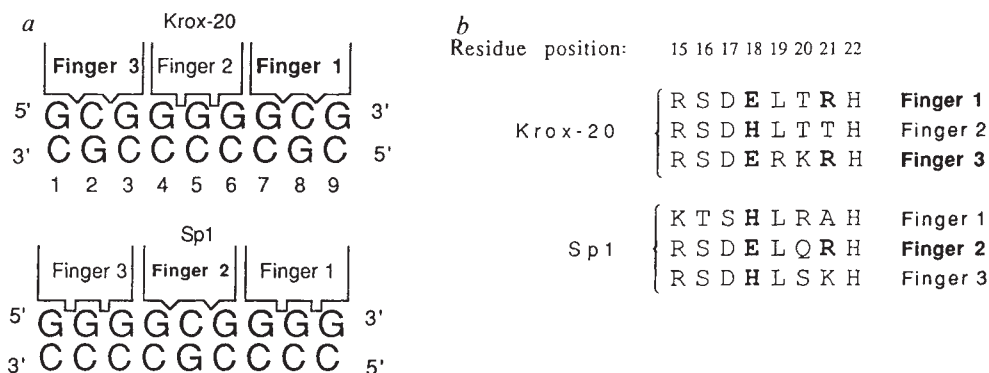
If the three zinc fingers of Krox-20 and Sp1 bind to their target sequences in the same way, then each is likely to span three base pairs (bp); two types of DNA specificity would then be feasible (Fig. 1a): Krox-20 fingers 1 and 3, and Sp1 finger 2 (type-1 fingers) could interact with the sequence 5'-GCG-3', while Krox-20 finger 2 and Sp1 fingers 1 and 3 (type-2 fingers) would interact with the sequence 5'-GGG-3' (Fig. 1a). Alignment of the amino-acid sequences of the different Krox-20 and Sp1 zinc fingers reveals a strong similarity in a region (residues 15–22 (ref. 12)) that forms part of an  $\alpha$  helix that might be involved in base recognition<sup>6,12–14</sup> (Fig. 1b). Two positions in this region show variations between types 1 and 2: at position 18, Glu (E in single-letter code) and His (H) are strictly associ-

TABLE 1 Quantification of gel retardation experiments

|            | Krox-20 (2HT) | 2ER | 2ET | 2HR | 1HT | 2QR |
|------------|---------------|-----|-----|-----|-----|-----|
| 5'GCGGGGCG | +++           | +/- | -   | +++ | +/- | +++ |
| 5'GCGGCGCG | -             | +++ | +/- | ++  | -   | +++ |
| 5'GGGGGGCG | ++            | -   | -   | ++  | +/- | ND  |
| 5'GCGGGGGG | +             | -   | -   | ++  | ++  | ND  |
| 5'GCGGCGCG | +++           | -   | ND  | ND  | ND  | ND  |
| 5'GCGCCGCG | -             | -   | ND  | ND  | ND  | ND  |
| 5'GCGGCGCG | -             | -   | ND  | ND  | ND  | ND  |

The proportion of oligonucleotide retarded as a result of complex formation was estimated by densitometer scanning of the autoradiograms (Fig. 2b, and data not shown). This value was then compared with the value corresponding to the binding of wild-type Krox-20 to its consensus target site. +++, Proportion of complex similar to that formed by Krox-20 with the sequence 5'-GCGGGGCG-3', within a 2.5-fold range; ++, proportion 2.5–5-fold lower than the control; +, proportion 5–20-fold lower than the control; +/-, proportion at least 50-fold lower than the control; minus sign indicates no binding detected. ND, not determined.

FIG. 1 Model for the interactions of Krox-20 and Sp1 with their respective consensus binding sites. *a*, Model showing each zinc finger spanning 3 bp; Krox-20 and Sp1 fingers are of two types: type-1 fingers (Krox-20 fingers 1 and 3, and Sp1 finger 2) and type-2 fingers (Krox-20 finger 2, and Sp1 fingers 1 and 3) which interact more specifically with the sequences 5'-GGG-3' and 5'-GCG-3' respectively. The relative orientation of proteins and DNA, originally arbitrary, is consistent with the mutagenesis analysis of finger 1 of Krox-20. *b*, Alignment of the amino-acid sequences (one-letter code)<sup>24</sup> of Krox-20 and Sp1 zinc fingers within the conserved region potentially involved in specific base recognition. Type 1 and type 2 zinc fingers are indicated



ated with types 1 and 2 respectively (Fig. 1b); at position 21, Arg (R) is associated with type 1 and there is variability in the case of type 2 (Fig. 1b). To establish whether these two positions are important for DNA recognition by Krox-20, we constructed several mutant derivatives of Krox-20. The relative affinities of the corresponding proteins for different DNA target sequences were estimated by gel retardation experiments<sup>15,16</sup>.

Initially positions 18 and 21 of the conserved region of Krox-20 finger 2 (originally His and Thr residues) were converted to Glu and Arg respectively (Fig. 2a). The resulting protein, Krox-20/2ER, did not bind to the Krox-20 consensus binding sequence 5'-GCGGGGGCG-3', but formed an abundant complex with an oligonucleotide carrying the sequence 5'-GCGGCGGCG-3'; this sequence was not recognized by the wild-type Krox-20 protein (Fig. 2b, and Table 1). Methylation interference experiments<sup>17</sup> indicated that methylation of any of the nine G residues of the mutant binding sequence interfered with the formation of the complex with Krox-20/2ER, whereas methylation of A or G residues located outside this sequence did not (data not shown). Oligonucleotides carrying other G-to-C transversions in the binding site were not recognized by Krox-20/2ER (Table 1). Thus the double amino-acid change resulted in a precise modification of the specificity of DNA recognition.

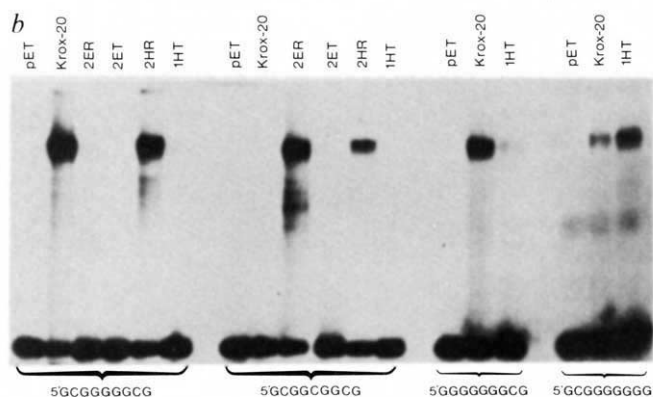
To delimit the contribution of each amino acid, we constructed two other finger-2 mutants with a single amino-acid modification at positions 18 or 21: Krox-20/2ET and Krox-20/2HR (Fig. 2a). Krox-20/2ET did not bind efficiently to any of our target oligonucleotides (Fig. 2b and Table 1). Krox-20/2HR bound not only to the Krox-20 binding site with an affinity similar to that of the wild-type protein (Fig. 2b and Table 1), but also to the mutant sequence 5'-GCGGCGGCG-3', albeit with a slightly reduced affinity compared with Krox-20/2ER. Thus both positions of Krox-20 finger 2 were involved in DNA specificity. To test our model further, we made a reciprocal construction, Krox-20/1HT, in which positions 18 and 21 of finger 1 were occupied by His and Thr (T) respectively (Fig. 2a). This protein was tried against several target sequences (Fig. 2b and Table 1), but bound significantly only to the sequence 5'-GCGGGGGGG-3'. This confirms the essential part played by amino acids 18 and 21 in the specificity of recognition. Also, these results indicate that the N-terminal finger of Krox-20 binds to the 3' part of the binding site and that each finger spans about 3 bp.

A Wilms' tumour candidate gene encodes a zinc-finger protein with four fingers, three of which are closely related to those of Krox-20 (ref. 18). The second finger contains a Gln (Q) at position 18 and an Arg at position 21. We tested the effect of

| <i>a</i> | Krox-20       | Krox-20/2ER   | Krox-20/2HR   | Krox-20/2ET   | Krox-20/2QR   | Krox-20/1HT   |
|----------|---------------|---------------|---------------|---------------|---------------|---------------|
| Finger 1 | R S D E L T R | R S D E L T R | R S D E L T R | R S D E L T R | R S D E L T R | R S D H L T T |
| Finger 2 | R S D H L T T | R S D E L T R | R S D H L T R | R S D E L T T | R S D Q L T R | R S D H L T T |

FIG. 2 Gel retardation analysis of the interactions between Krox-20 derivatives and potential target sequences. *a*, Amino-acid sequences of the conserved regions of fingers 1 and 2 of Krox-20 derivatives in which modifications, shown in bold, have been introduced. *b*, Gel retardation analysis. The nucleotide sequence of the central part of each oligonucleotide is indicated. The bacterial extracts contained the following Krox-20 derivatives: pET, control bacterial extract containing no Krox-20 derivative; Krox-20, wild-type Krox-20; 2ER, Krox-20/2ER; 2ET, Krox-20/2ET; 2HR, Krox-20/2HR; 1HT, Krox-20/1HT.

**METHODS.** Mutations were introduced into a Krox-20 cDNA sequence<sup>9</sup> by polymerase chain reaction using oligonucleotide primers containing the desired mutations. The primers covered the following parts of the gene, numbered as in ref. 11: (1) Krox-20/2ER: positions 1,038 to 1,083; codon changes, CAC to GAA and ACT to AGA. (2) Krox-20/2ET: positions 1,038 to 1,081; codon change, CAC to GAG. (3) Krox-20/2HR: positions 1,038 to 1,083; codon change, ACT to CGT. (4) Krox-20/1HT: positions 1,049 to 978; codon changes, GAG to CAT and AGG to ACG. Polymerase chain reaction was performed as described<sup>25</sup>. The amplified mutated DNA fragments were used to replace the wild-type fragment in the expression plasmid pET-Krox-20 (ref. 9) and their complete nucleotide sequences were established. pET-Krox-20 is a derivative of pET3a (refs 26, 27), where a Krox-20 complementary DNA is under the control of the T7 promoter. Expressed proteins were isolated from bacteria transformed with pET3a or derivatives of pET-Krox-20 (ref. 9) and quantitated by western blotting<sup>28</sup> using a rabbit



polyclonal antibody directed against a bacterial Krox-20 fusion protein (data not shown). In gel retardation experiments<sup>9</sup> we used the same amount of Krox-20 derivative proteins. The Krox-20 binding site was obtained by hybridization of the oligonucleotides 5'-CTCTGTACGCGGGGCGGTTA-3' and 5'-CTCTAACCGCCCGCGTACA-3'. Other target sequences were derived by modification of the central sequence as discussed.



this combination on recognition by Krox-20 finger 2 (Fig. 2a): Krox-20/2QR interacted with high affinity with both the wild-type Krox-20 binding site and the mutant sequence 5'-GCGGCGGCG-3' (Table 1). This indicates that fingers 2-4 of the Wilm's tumour gene product might interact with the sequence 5'-GCGGGGG<sup>C</sup>/G-G-3'.

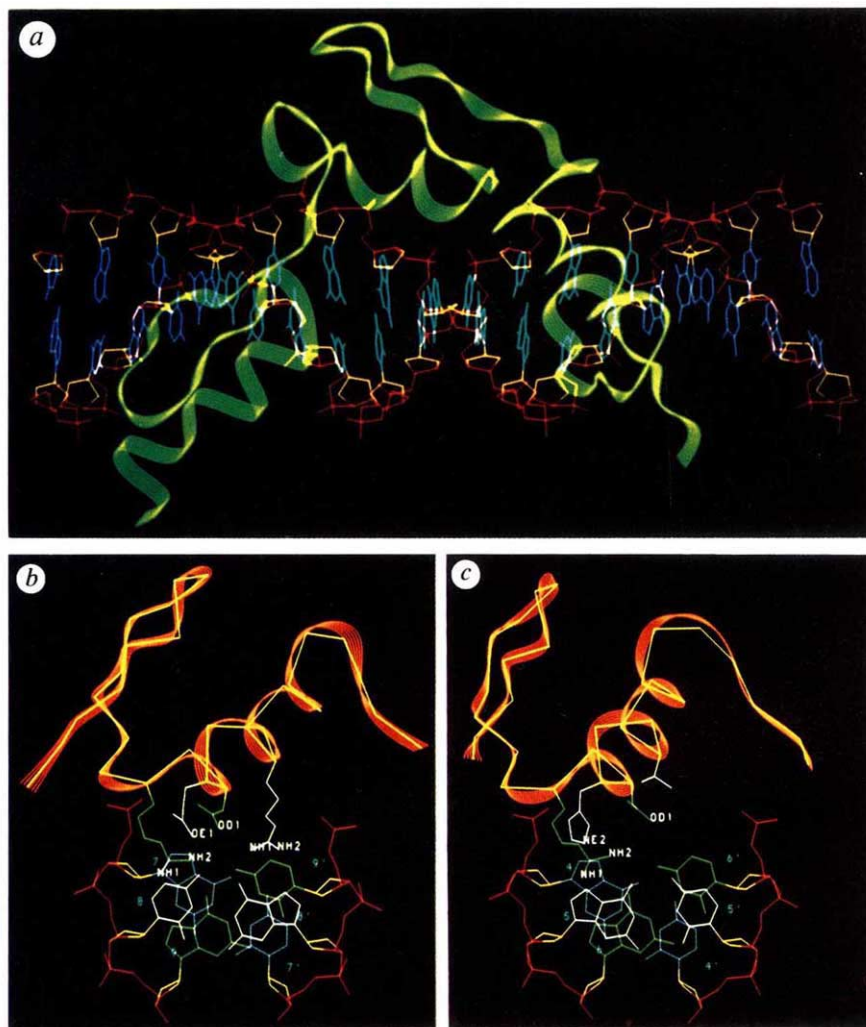
Using the structure determined by NMR for a single zinc finger<sup>6</sup> as a framework for molecular graphics, we docked the three fingers of Krox-20 with a 17-bp B-form DNA fragment representing the experimental oligonucleotide (Fig. 3a). Side chains at positions 18 and 21 of fingers 1 and 2 can be brought close to base pairs 8 and 5 respectively (see Fig. 1a for numbering of base pairs), in agreement with our mutagenesis data. Just four of the  $\alpha$  helix side chains on each finger, residues 15, 17, 18 and 21, are in a position to provide base-specific ligands. By analogy with bacterial repressors<sup>19,20</sup>, we tested these four candidate base-specific residues for their potential to hydrogen bond with the two donor sites on guanine or the single acceptor site on cytosine<sup>21</sup>. In finger 1 (Fig. 3b), the carboxyl of E-18 could accept an H-bond from C-8 in the DNA and R-21 could donate up to two H-bonds to G-8' in the complementary strand. Two additional possibilities for finger 1 would be to form two H-bonds from R-15 to G-9, and to accept one H-bond by D-17 from the complementary base C-9'. In finger 2 (Fig. 3c), R-15 could donate two H-bonds to G-6, possibly also recognizing G-7 should bifurcation of the H-bonds occur or the guanidinium group be positioned orthogonally to the bases, so providing one

bond to each guanine N7 atom. The carboxyl of D-17 is able to accept an H-bond from C-6', and possibly a second H-bond from the neighbouring C-7' if the DNA is distorted from ideal B form. H-18 could donate an H-bond from the NE1 to the N7 atom of G-5. T-21 cannot reach any bases directly, which is consistent with the lack of conservation of this residue in the type-1 finger. Finger 2 is unable to provide ligands to base pair 4, consistent with the minimal discrimination found at this pair<sup>9</sup>.

In conclusion, our results indicate that Krox-20 zinc finger positions 18 and 21 combine to discriminate a single base pair by direct contact. Single exchange at either position tends to weaken both affinity and selectivity whereas substitution of Glu by Gln (which is both a donor and an acceptor of H-bonds) at position 18 destroys the selectivity of the position (Table 1). In finger 2, position 18 is the strongest determinant of specificity for base pair 5, consistent with its buried position in the docked model. Position 21 is not so deeply buried and, at least as Arg, contributes more to binding affinity than specificity, given the low discrimination of Krox-20/2HR and Krox-20/2QR for base pair 5. It has been suggested that zinc fingers span either 5.5 bp, on the basis of periodicity in recognition sites<sup>22</sup>, or 2-3 bp, from molecular modelling<sup>12,23</sup>. Our data indicate that each Krox-20 finger spans 3 bp, providing base discrimination to either 2 or 3 bp. The ratio of 2 or 3 bp per finger should apply to other finger proteins too. Although longer linkers between fingers would allow more base pairs to be spanned, it is unlikely that more than 3 bp could be recognized by individual fingers because

FIG. 3 Modelling of the interaction of Krox-20 and DNA. **a**, Krox-20 zinc fingers docked to 17 bp of DNA viewed to show the backbone path of finger 2. Fingers go from right to left, the core binding-site sequence 5'-GCGGG-GGCG-3' from left to right. Each finger spans 3 bp, with its  $\alpha$  helix lying in the major groove. The protein backbone is shown as a green ribbon. The DNA backbone is coloured red and yellow. The Krox-20 recognition site bases are turquoise, the others are blue. **b**, Finger 1 backbone and four possible base-discriminatory side chains, docked with bp 7-9 (sequence GCG), viewed axial to the DNA. The four amino acids can reach bp 8 and 9 but not 7. Interacting residues and bases are coloured the same: bp 9 (G.C), closest to the viewer, and side chains R-15 and D-17 are green; bp 8 (C.G) and side chains E-18 and R-21 are white; bp 7 (G.C) is blue. The peptide backbone is shown by a yellow C $\alpha$  trace and a red ribbon. Side chain H-bond donor or acceptor atoms are labelled: NH1 and NH2 from R-15 and R-21; OD1 from D-17; OE1 from E-18. **c**, Finger 2 docked with base pairs 4-6 (sequence GGG). Neither T-21 nor bp 4 make contacts. Base pair 6 (G.C) and side chains R-15 and D-17 are green; bp 5 (G.C) and side chains H-18 and T-21 are white; bp 4 (G.C) is blue. NE2 is from H-18. Other colorations and labelling of side chains are as in **b**.

**METHODS.** Three copies of the zinc finger structure, as determined by NMR<sup>6</sup>, were concatenated and the side chains substituted with the Krox-20 sequence. Krox-20 finger 1 was docked into the major groove of the DNA such that residues E-18 and R-21 were close to bp 8. Three conserved positive charges at positions 2, 11 and 24 were able to reach phosphates at nucleotides 11', 6 and 5 respectively. Using rotation about the backbone dihedral angles of the linker between fingers 1 and 2 (residues TGHKPF), the second finger was positioned so that residues H-18 and T-21 were close to bp 5. Conserved positive side chains of finger 2 could reach phosphates at nucleotides 8', 3 and 2. Similarly, finger 3 was centred on bp 2, contacting phosphates 5', -1 and -2. Finally, side chain and fine linker adjustments positioned polar atoms within approximate H-bonding distance to the bases ( $3 \pm 1$  Å). Software EMBLFRDO (H. Boss-



hard, modified from ref. 29) was used for docking, and INSIGHT<sup>30</sup> for preparing the figures on an Evans and Sutherland PS390 graphics monitor.

only four side chains are in a position to ligand to bases. Examination of other zinc-finger proteins<sup>12</sup> reveals that the positions responsible for discrimination are highly variable, so extension of our detailed suggestions beyond the Krox-20/Sp1 family must await further experiment. □

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## Three-dimensional structure of the *E. coli* DNA-binding protein FIS

Dirk Kostrewa, Joachim Granzin, Christian Koch\*, Hui-Woog Choe, Srinivasan Raghunathan, Wojciech Wolf, Jörg Labahn, Regine Kahmann\* & Wolfram Saenger

Institut für Kristallographie, Freie Universität Berlin, Takustrasse 6, D-1000 Berlin 33, Germany

\* Institut für Genbiologische Forschung Berlin GmbH, Ihnestr. 63, D-1000 Berlin 33, Germany

THE factor for inversion stimulation, FIS, is involved in several cellular processes, including site-specific recombination and transcriptional activation<sup>1-4</sup>. In the reactions catalysed by the DNA invertases Gin, Hin and Cin, FIS stimulates recombination by binding to an enhancer sequence<sup>1</sup>. Within the enhancer, two FIS dimers (each 2 × 98 amino acids)<sup>5-7</sup> bind to two 15-base-pair consensus sequences<sup>8,9</sup> (Fig. 1) and induce bending of DNA<sup>10,11</sup>. Current models propose that the enhancer-FIS complex organizes a specific synapse, either through direct interactions with Gin, or by modelling the substrate into a configuration suitable for recombination<sup>1,9,12</sup>. Using X-ray analysis at 2.0 Å resolution, we now show that FIS is composed of four α helices tightly intertwined to form a globular dimer with two protruding helix-turn-helix motifs. The 24 N-terminal amino acids are so poorly defined in the electron density map as to make interpretation doubtful,

indicating that they might act as 'feelers' suitable for DNA or protein (invertase) recognition. We infer from model building that DNA has to bend for tight binding to FIS.

We crystallized FIS<sup>1</sup> from 1.2-1.5 M phosphate<sup>13</sup> and determined the crystal structure by multiple isomorphous replacement (MIR) combined with solvent flattening<sup>14,15</sup>, supported by phase combination from partial structure information with MIR phases<sup>16</sup> (Table 1). Two molecules (a dimer) occupy the crystal asymmetric unit. Refinement<sup>17</sup> of the 2 Å-resolution model is in progress with an *R*-factor of 25%. The electron density is well defined, except for the N-terminal 24 amino acids in both molecules, for which only partial, discontinuous density is present.

The two monomers in the asymmetric unit are related by non-crystallographic twofold rotation symmetry (Fig. 2). This is a structural characteristic of the FIS dimer, as averaging procedures were deliberately avoided during structure determination and refinement. Main secondary structure elements are four α helices A, B, C, D. The polypeptide chain begins abruptly with Lys 25, which we correlate with the helix-breaking character of the adjacent Pro 26. The loops between helices A, B and B, C are type I' β turns<sup>18</sup>, with glycine in position 3 as required for steric reasons. The C terminus of FIS and the C terminus of helix C are folded into type I β turns (Fig. 1).

The dimer has an ellipsoidal shape, 25 × 35 × 55 Å, with two protrusions formed by helices D<sub>I</sub>, D<sub>II</sub>. Helices A<sub>I</sub>, A<sub>II</sub>, B<sub>I</sub>, B<sub>II</sub> are antiparallel and arranged in a four-helix bundle stabilized predominantly by hydrophobic forces and by Van der Waals' interactions. In addition, the dimer is stabilized by four main-chain hydrogen bonds connecting the N terminus of helix B in one monomer with the C terminus of helix C in the other monomer.

We ensured that the ill-defined electron density for the N-terminal 24 amino acids was not due either to the solvent flattening procedure or to limited proteolysis (data not shown). Hence it must be associated with local disorder, which we attribute to segmental, dynamic flexibility of the N terminus. This is an intrinsic property of the FIS dimer and could be of importance for its biological function.

All the transcriptional regulatory proteins studied by crystallography<sup>19</sup> are dimeric to match the twofold rotational symmetry of DNA. With the exception of the Met<sup>20</sup> and Arc<sup>21</sup> repressors, they all have a 21-amino-acid helix-turn-helix motif in common<sup>22</sup>. The second α helix specifically recognizes B-form DNA by inserting into successive major grooves<sup>19</sup>. In FIS, segment 74 to 93 is folded as in the other helix-turn-helix motifs, with an r.m.s. deviation of only 0.51 Å if the Cα positions are superimposed on the corresponding amino acids 16-35 of λ cro repressor<sup>22,23</sup> (data not shown). From a survey of 37 related proteins<sup>22</sup>, position 9 of the motif must be occupied by a glycine

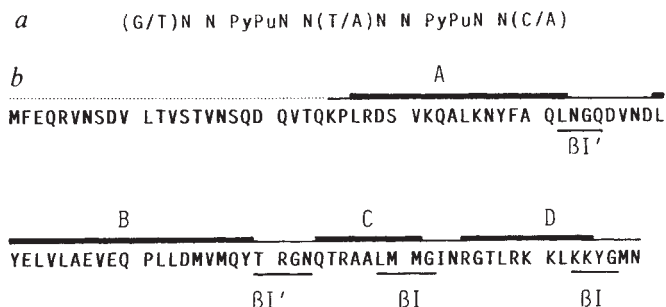


FIG. 1 a, Consensus sequence of the FIS binding site; b, amino-acid sequence (single-letter code) of FIS grouped in blocks of ten residues. Dotted lines indicate the disordered N terminus of the molecule; α helices A-D shown by solid lines; β turns marked by BI', BI.

TABLE 1 Crystallographic and MIR data

| Data set                                  | Space group $P2_12_12_1$ ( $a=47.39(5)$ , $b=50.98(6)$ , $c=79.67(2)$ Å) |                                 |                                                                     |                                                                                 |
|-------------------------------------------|--------------------------------------------------------------------------|---------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------------------------|
|                                           | Native                                                                   | UO <sub>2</sub> Cl <sub>2</sub> | [PtCl <sub>2</sub> (C <sub>2</sub> H <sub>4</sub> ) <sub>2</sub> ]* | [PtCl <sub>2</sub> (C <sub>2</sub> H <sub>4</sub> ) <sub>2</sub> ] <sup>†</sup> |
| No. of collected reflections              | 8,221‡(-)                                                                | 4,136 (-)                       | 3,595 (-)                                                           | 23,255 (8.6%)                                                                   |
| $R_{\text{sym}}$ ( $I$ ) in parentheses§  | 44,050‡ (6.4%)                                                           |                                 |                                                                     |                                                                                 |
| No. of unique reflections                 | 12,847 <sup>  </sup>                                                     | 3,907                           | 3,053                                                               | 4,648                                                                           |
| Maximum resolution in (Å)                 | 2.0                                                                      | 3.0                             | 3.3                                                                 | 2.8                                                                             |
| Mean fractional isomorphous change in (%) | —                                                                        | 16.7                            | 23.3                                                                | 33.9                                                                            |
| No. of heavy atom sites                   | —                                                                        | 3                               | 4                                                                   | 4                                                                               |
| Overall phasing power                     | —                                                                        | 1.08                            | 2.36                                                                | 2.17                                                                            |

Phasing power is  $F_n/E_{r.m.s.}$ , where  $F_n$  is heavy-atom structure factor and  $E$  is residual lack of closure.

\* Data set collected on STOE four-circle diffractometer, sealed tube X-ray generator.

† Native data set and anomalous data set collected on image-plate area detector at the EMBL outstation, Deutsches Elektronen Synchrotron, Hamburg.

‡ Native data set collected on ENRAF-Nonius Turbo-CAD4 four-circle diffractometer, rotating anode X-ray generator FR571.

§  $R_{\text{sym}} = \frac{\sum_i |I_i - \bar{I}|}{\sum \bar{I}}$ ; with  $I_i$  individual and  $\bar{I}$  averaged intensities native data set collected as described in second footnote.

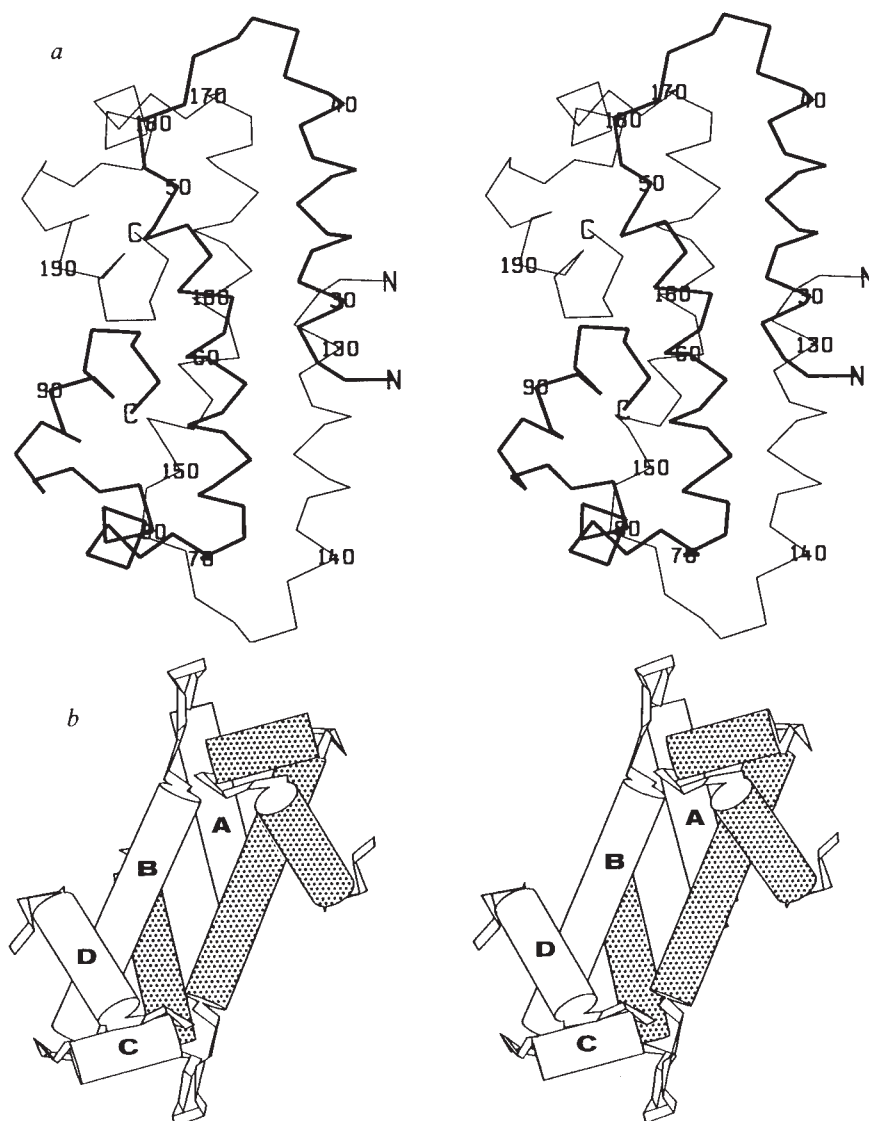
|| Merged native data set ‡§;  $R_{\text{merged}} = 2.3\%$  ( $R_{\text{merged}} = \sum \Sigma_i |\bar{F} - F_i| / \sum \Sigma F_i$ ).

(Gly 82), amino acids at positions 4 and 15 are uncharged (Ala 77 and Leu 88), and the one at position 5 is unbranched (Ala 78). In contrast to the other proteins whose recognition helix has either no or only a few positive charges, helix D in FIS contains six positively charged residues (two Arg and four Lys). This could indicate that FIS recognizes DNA through

interactions that are predominantly nonspecific, an assertion that does not contradict the degenerate FIS consensus binding site<sup>8</sup> (Fig. 1).

Footprinting analyses have shown that FIS protects bases in two successive major grooves on the DNA helix<sup>9,24</sup>. The recognition helices D<sub>I</sub>, D<sub>II</sub> seem to be in a suitable orientation for this

FIG. 2 Stereo views of the FIS dimer structure. *a*, C $\alpha$  trace, with every tenth residue numbered. Amino acids 1 to 98 are molecule I (thick trace), 101 to 198 are molecule II (thin trace). The 24 N-terminal residues are not seen in the electron density, N termini (amino acid 25) and C termini are indicated by corresponding letters. To the right of the stereo view, the four-helix bundle ( $\alpha$  helices A<sub>I</sub>, A<sub>II</sub>, B<sub>I</sub>, B<sub>II</sub>) is in an upright orientation, to the left are the helix-turn-helix motifs, helices C<sub>I</sub>, D<sub>I</sub> and C<sub>II</sub>, D<sub>II</sub>. The view is perpendicular to the local twofold axis which is oriented horizontally. *b*, View along the twofold helix onto the helix-turn-helix motif, helix D being the 'recognition' helix. Helices in molecule I are denoted A to D; those in molecule II are shaded.





kind of interaction with DNA. This is also indicated by single point mutations in helix D of FIS (Arg 85 Val and Lys 91 Glu) which interfere with DNA binding (C.K. and R.K., unpublished results).

Model building with straight B-form DNA allowed us to fit the D helices into the major grooves only through their N termini; the C termini were exposed to solvent so that most of the positive charges were not in contact with DNA phosphates. This is because the vertical separation between the D helices of  $\sim 24$  Å is too short to match ideally with the 29 Å separation between the bottoms of adjacent DNA major grooves. (The distance between successive major grooves along the DNA double helix is 34 Å. As the major grooves are inclined by  $58^\circ$  against the axis, the vertical (or shortest) distance between the bottoms of the major grooves is  $34 \text{ Å} \times \sin 58^\circ = 29 \text{ Å}$ .)

There are solutions to this dilemma. One is to invoke substantial conformational changes within the FIS dimer upon binding to DNA; the other is to bend the DNA in the complex with FIS, as has been indicated previously<sup>10,11</sup>. The DNA bending angle for a particular FIS site has been estimated as  $\sim 90^\circ$  (ref. 11). Assuming this DNA curvature has a radius of 43 Å, as in the nucleosome core particle<sup>25</sup>, the bent DNA was docked with the FIS dimer (Fig. 3). The fit is superior to that with straight DNA. The recognition helices D<sub>I</sub>, D<sub>II</sub> are now inserted in adjacent major grooves, and the positively charged Arg and Lys side chains can interact with the DNA phosphates. The modelled complex is consistent with the available footprint data which have shown that FIS protects guanines against methylation at N(7) in two successive major grooves. Sites across the minor groove on the back of the complex become hypersensitive to DNaseI (refs 9, 24), which correlates with an altered minor groove width and with bending of the DNA towards the major groove<sup>19</sup>. Further evidence in support of this model is provided by the position of Gln 74; it is located so that the amide group can hydrogen-bond with a DNA-phosphate oxygen, as in repressors  $\lambda$ , 434 and Trp, which also have Gln in this position<sup>26</sup>.

Our model does not exclude the possibility that the flexible N-terminal parts of the FIS dimer make additional DNA contacts and have 'feeler'-like functions that parallel those proposed for the C-terminal part of cro<sup>23</sup>, the N-terminal part of  $\lambda$  repressor<sup>27</sup>, and the distal parts of the DNA-binding arms of the HU-protein<sup>28</sup>. Through its flexible sections, the FIS-DNA complex possibly recognizes another partner—either a different site on the DNA or another protein (perhaps a recombinase). This idea is attractive in view of the proposed specific organization of the synaptic complex by the FIS-enhancer complex during recombination<sup>1,9,12</sup>. □

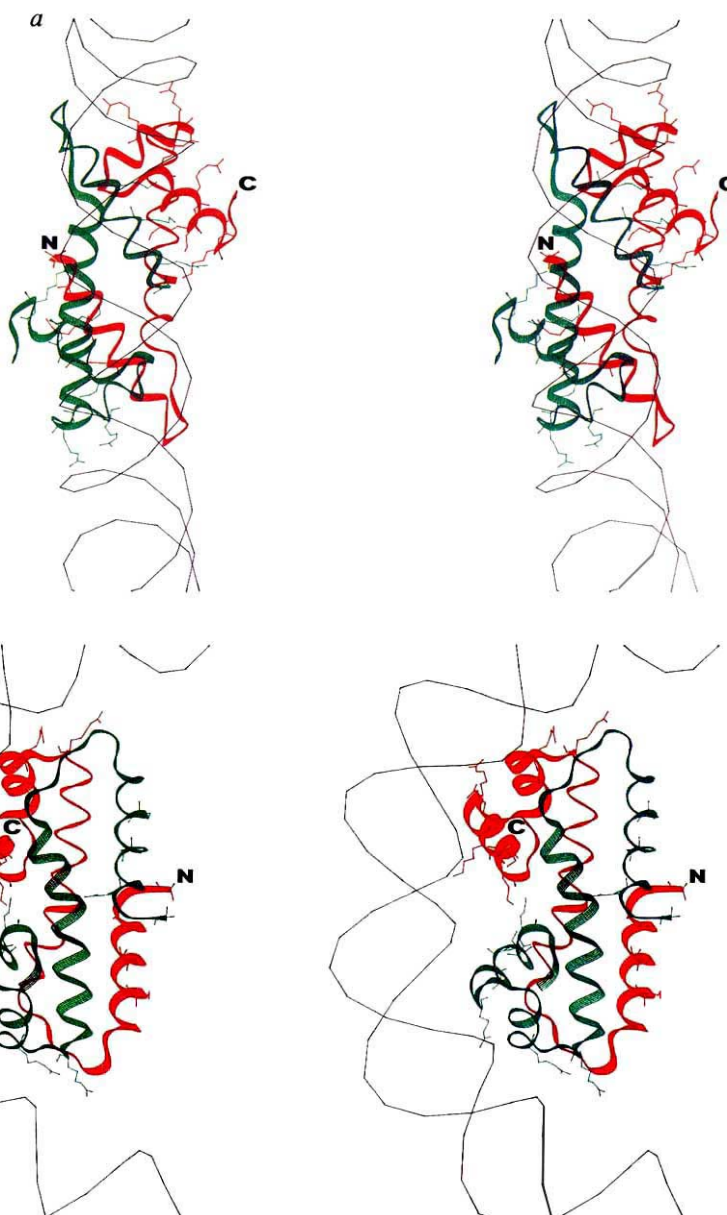


FIG. 3 Views of the modelled FIS-DNA complex. Positively charged amino acids (Lys, Arg) are indicated with their side chains. DNA is shown only as a trace through P atoms, drawn with program SUPERHELIX (H. H. Ohlenbusch, personal communication). DNA is bent as in the nucleosome core, radius 43 Å. The recognition helices D of the helix-turn-helix motif are inserted in successive DNA major grooves. *a*, View along the twofold axis of the complex; *b*, view with the twofold axis horizontally. The twofold symmetry is only approximate as FIS coordinates were taken directly from this analysis without averaging.

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